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13. ABSTRACT (Maximum 200 Words)

CD95 mediated apoptosis is important for numerous processes, and often tumor cells develop a resistance to CD95 mediated apoptosis. MCF7-Fas-Bcl-x_L cells, which are a model of a breast carcinoma cell, are protected from CD95 mediated apoptosis by binding and inactivating active caspase-8 generated at the DISC on mitochondria. The mechanism of this binding and its functional relevance is the subject of the study. The binding properties and mechanism of the subsequent degradation is addressed. Additionally, CD95 signaling in these apoptosis resistant tumor cells was determined to activate numerous signaling pathways, resulting in the activation of multiple genes including anti-apoptotic, pro-proliferative, and pro-tumorigenic genes, and ultimately an increase in motility and invasiveness. This increase was determined to be dependent on the activation of the NF-κB pathway, as well as the Erk1/2 pathway, and interestingly, caspase-8 activity. These observations are profound and relevant to the study of numerous breast cancers, and my redefine the way in which CD95 signaling is viewed. It also addresses the role of caspase-8 in breast carcinoma cells, which may induce tumor progression in certain circumstances rather than the death of the cell.

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Caspase-8, CD95, apoptosis, Bcl-x_L, BAR, NF-κB

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INTRODUCTION

Apoptosis is a physiologically important form of cell death that is used to clear cells following numerous processes, such as the clearance of immune cells following an immune response (1). Additionally, immune surveillance, involved in eliminating tumors is mediated by apoptosis, typically by tumor infiltrating lymphocytes (*2*). It is well established that most if not all tumor cells develop mechanisms to avoid an apoptotic response, indeed it is believed that tumor cells must develop this resistance to further progress (*3*). Therefore it is an important task to determine the mechanism of how tumor cells develop this resistance in an effort to ultimately break the resistance and induce apoptosis in tumor cells. One of the mechanisms of apoptosis that is important for tumor elimination is by the activation of the death receptor CD95 (*2*). This receptor contains protein-protein interaction domains that when the receptor is ligated, recruit molecules that ultimately result in the activation of cysteine aspartate proteases (caspases) resulting in the cleavage of proteins and dismantling of the cell (*4, 5*). Initially, caspase-8 is activated following CD95 stimulation, followed by the activation of other executioner caspases (*6, 7*). In many tumor cells this apoptotic signaling is blocked. In many cells, including the breast carcinoma cell MCF7, expression of the anti-apoptotic protein Bcl-x_L can block CD95 mediated apoptosis (*8, 9*). The mechanism of this apoptosis resistance was found to be mediated by the binding and inactivation of caspase-8 on the mitochondria (*9*). Interestingly, however it was determined that the entire pool of caspase-8 was activated in these cells. This phenomenon is the basis for this research project. The mechanism and functional outcome of this binding is addressed in MCF7 cells as well as numerous other breast cancer cell lines. Ultimately, this research would be applied to the treatment of breast cancer by attempting to overcome apoptosis resistance. Additionally, new data expose a striking and groundbreaking observation that the death receptor CD95 may not always behave as a death receptor but may activate other non-apoptotic pathways following stimulation. This phenomenon was observed in a number of breast cancer cell lines and appears to be a general one.

BODY

BAR expression in other cell lines: Characterization of anti-BAR antibodies

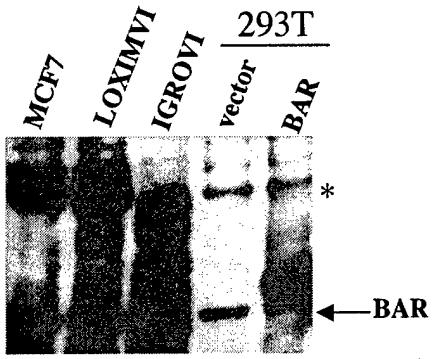

Figure 1. Cells as indicated were lysed and lysates subjected to Western blot analysis using anti-BAR antibody clone 9B5. * indicates non-specific band. 293T cells were transfected with BAR expression vector.

In order to examine the effects of BAR on the binding of caspase-8 to mitochondria, reagents to examine BAR are absolutely essential. To this end, we generated mouse anti-BAR monoclonal antibodies and rabbit anti-BAR polyclonal antibodies. These antibodies were efficient in detecting BAR when ectopically expressed in 293T cells. Additionally, we determined the efficacy of detection of endogenous BAR by performing Western blots on lysates from cells known to express high levels of BAR (Figure 1) (*10*). These data demonstrated that the mouse anti-BAR antibodies were able to detect endogenous BAR, indicating that these antibodies are useful in the characterization of BAR as it is expressed at endogenous levels in cells. Additionally, transfected 293T cells were used to test the panel of anti-BAR antibodies. Each of these antibodies detected BAR efficiently (Figure 2).

The determination of binding partners of BAR would require the ability to selectively isolate BAR. To this end, we performed immunoprecipitation (IP) assays using the anti-BAR antibodies in different combinations to determine how efficient the IP and detection of BAR was using our antibodies. These experiments indicate that our panel of antibodies is quite efficient at isolating BAR using different combinations of the antibodies (Figure 3). This result indicates that these antibodies will be of great use in the isolation of BAR itself as well as associated proteins. Additionally, the antibodies could be used to isolate endogenous BAR and any associated proteins. Since this panel of antibodies is able to IP BAR, they could potentially be used for immunofluorescence as proposed in the original statement of work.

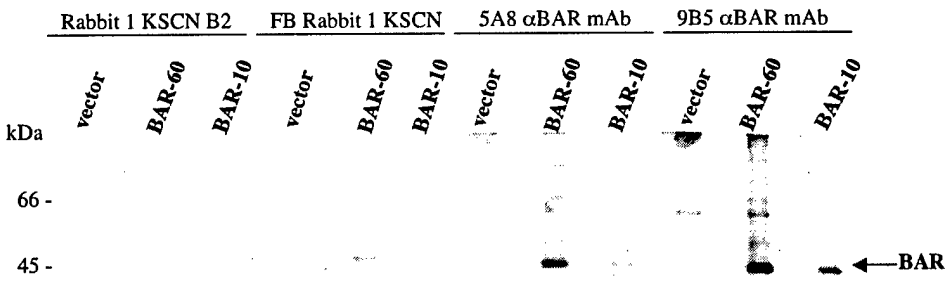

Figure 2. Lysates from 293T cells transfected with vector control or BAR expression vectors (with 60 or 10 μg of protein from lysed cells). Lysates were probed with indicated antibodies.

Finally, as an independent method of determining the of BAR interaction with caspase-8, sucrose gradient centrifugation was performed. In this experiment, cells lysates were layered on discontinuous sucrose gradients to separate interacting proteins. It was determined that these experiments resulted in the detection of caspase-8 in a fraction which implies a complex greater than monomeric caspase-8. Interestingly, BAR was detected in this fraction as well, indicating that it is likely that BAR and caspase-8 interact in a complex that can be separated by sucrose centrifugation (data not shown). This experimental design can be used to examine interacting partners with BAR, and can ultimately be used in experiments using transfected BAR mutants to determine the interacting domains required for the interaction with and inactivation of caspase-8.

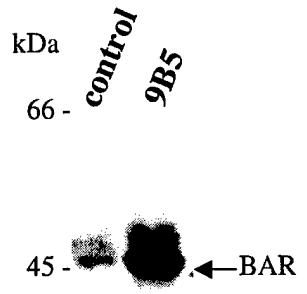

Figure 3. 293T cells were transfected with BAR expression vector, and IP performed with control or 9B5 mouse antibody followed by detection with the indicated rabbit antibody.

CD95 stimulation of apoptosis resistant breast cancer cells results in the activation of alternative, non-apoptotic pathways

The expression of CD95 on MCF7 cells results in a CD95 apoptosis sensitive phenotype (*8, 11*). MCF7 cells transfected with both CD95 (Fas) and Bcl-x_L (MCF7(FB)) however are CD95 apoptosis resistant (*8, 11*). We determined as described in the original proposal, that this resistance was due to the binding and inactivation of caspase-8 on the surface of mitochondria mediated by the concomitant interaction with both Bcl-x_L and BAR (*9*). Interestingly, despite the fact that these cells were completely resistant to CD95 mediated apoptosis, the entire cellular pool of caspase-8 was activated following stimulation. It has also recently been reported that caspases may be involved in processes aside from apoptosis. It was observed that caspase inhibitors block the migration of

certain cells (*12*), that inhibitors of caspases may also block the proliferation of T cells (*13, 14*). Also, CD95 stimulation has been reported as a costimulator of T cells (*15*), and T cells deficient in caspase-8 have a block in proliferation as well (*16*). While performing experiments to examine the outcome of caspase-8 inhibition following stimulation through CD95, I examined possible alternative outcomes of CD95 signaling when caspase-8 is effectively inhibited in breast carcinoma cells. These outcomes could involve the binding of caspase-8 to mitochondria, either directly affecting cellular metabolism, or indirectly by allowing cells to survive caspase-8 production following CD95 stimulation, and thereby activating other pathways, or caspase-8 could have a direct role in alternative signaling pathways emanating from the CD95 receptor. To examine alternative CD95 signaling experimentally, I determined the activation of genes following CD95 stimulation. In these experiments I utilized the MCF7(FB) cells that were the model system used in the publication leading to this proposal. These cells serve as a good model for a breast cancer that is relatively highly CD95 positive, but that is resistant to CD95 mediated apoptosis due to the expression of Bcl-x_L. Interestingly, CD95 stimulation in these apoptosis resistant MCF7(FB) cells resulted in an activation of numerous genes, as assayed by gene chip analysis (*17*). We utilized the Affymetrix U133 gene chip set, which covers approximately 33,000 human genes. The genes activated by CD95 stimulation in MCF7(FB) cells are listed in Table 1. A number of these genes are known anti-apoptosis genes, as well as genes involved in proliferation, and tumor progression. Finally, numerous genes that were activated are known targets of the transcription factor NF-κB (marked with an asterisk in Table 1). This well studied transcription factor has been described primarily as an activator of anti-apoptotic genes, and has been implicated in the progression of tumors (*18*), including breast cancer [ref]. Additionally, to examine the effects of caspase-8 inhibition on gene activation I performed a gene array analysis on gene activation with cells that were preincubated with the caspase-8 selective inhibitor zIETD-fmk. This resulted in the activation of a much more limited number of genes, shown in bold in Table 1. Following this observation, I decided to examine more closely the effects of CD95 stimulation in apoptosis resistant tumor cells, using the MCF7(FB) cell as a primary model. The observations that follow are clearly applicable to breast cancer in general. This is especially true since it has been well established that NF-κB activation is one of the major mechanisms in breast cancer tumorigenesis [ref]. Therefore the activation of this signaling pathway could result in a more aggressive breast cancer. The activation of NF-κB could also be a result of the inactivation of caspase-8 on the mitochondria by BAR and Bcl-2 family members. The ultimate outcome of the observations are profound and may have implications in many tumor situations. These implications will be detailed below. Please see the attached manuscript referenced as (*17*) for detailed description of the following experiments.

CD95 stimulation of apoptosis resistant breast cancer cell line MCF7(FB) results in an increase in motility and invasiveness

Since numerous genes that were activated in the stimulated MCF7(FB) cells have been implicated in tumor progression, the potential tumor inducing activity of CD95 was tested. To examine this I utilized a well established *in vitro* assay for invasiveness, the Boyden chamber assay. In this assay, cells are plated in the top of two chambers, which contains a membrane with 8μm pores coated with the basement membrane mimetic Matrigel, with a chemoattractant in the bottom chamber (here 10% fetal calf serum) and stimulated (here with CD95 stimulus in both the top and bottom chambers). After the incubation cells which had not invaded are mechanically removed from the top of the membrane, and cells that have invaded fixed stained and counted. CD95 stimulation of MCF7(FB) breast carcinoma cells resulted in a profound increase in

invasiveness (*17*). This was observed with stimulation with both agonistic antibodies as well as CD95L. It was also observed in cells of multiple origins. Finally, CD95 induced migration (Boyden chamber without Matrigel coating) invasiveness was observed in cells stimulated with membrane bound CD95L, and with activated T cells (all in MCF7(FB) cells) indicating that cells stimulated through CD95 from multiple sources results in an increased *in vitro* invasiveness (*17*). This observation is of profound importance, since it is the first report of a tumor cell responding to CD95 stimulation with an activity that could result in an increased tumorigenicity. This observation is highly relevant for breast cancer research, since the cells used primarily in this study are a model for breast cancer.

CD95 stimulation of the breast cancer cell line MCF7(FB) results in the activation of the NF-κB and MAPK pathways

Since the gene chip experiments demonstrated that MCF7(FB) cells respond to CD95 stimulation with the activation of numerous genes known to be regulated by NF-κB, I examined the ability of CD95 stimulation to directly activate NF-κB in these cells. Using EMSA, as well as by reporter gene activity (CAT assay) it was determined that these cells responded to CD95 stimulation with profound NF-κB activation (*17*). The cells responded to CD95 stimulation with increased activation of all three major MAPK pathways as well, Erk1/2, p38, and JNK1/2 (*17*). This indicates that CD95 stimulation of the breast cancer cell line MCF7(FB) results not in apoptosis but in pathways that have been implicated in survival and increased tumorigenicity [ref]. It was also determined that NF-κB and Erk1/2 activation was required for the invasiveness observed in these cells following CD95 stimulation. Because we determined that the mechanism of inhibition of apoptosis in these cells is through the inactivation of caspase-8, we examined the requirement for caspase-8 activation for these processes. These cells did not require the activation of caspase-8 for the activation of any of the observed pathways, but the activation of caspase-8 was required for the invasive process (*17*). The level at which caspase-8 is required for these processes is not known. This result is highly interesting because it implicates caspase-8 in this CD95 mediated invasive process. This role of caspase-8 could potentially be a therapeutic target, and could both block invasiveness as well as sensitize the cell for apoptosis as proposed in the original statement of work. Because of the profound implications of these observations this line of experimentation was followed after the determination that caspase-8 inactivation by Bcl-x_L and BAR resulted in the survival of MCF7(FB) cells. Since this mechanism of apoptosis resistance may be applicable generally to breast cancer (or likely is involved in other breast cancer resistances) I have examined more closely the effects of CD95 stimulation on cells which use this mechanism to render themselves resistant to CD95 mediated apoptosis. I have attached a manuscript that has developed from the study of the MCF7(FB) model described in the original statement of work, which is currently being revised for resubmission to the EMBO Journal. These findings may have implications that are far reaching for tumor therapy and treatment. Indeed, if CD95 stimulation on apoptosis resistant tumor cells (and most tumor cells have developed some form of resistance to CD95 mediated apoptosis) results in the induction of a pro-survival, proproliferative, and pro-tumorigenic program, ultimately it may be advantageous to block CD95 signaling rather than promoting it. The promotion of CD95 mediated apoptosis in tumor cells is currently being considered as a method of potential therapy. These observations and this model become more striking when it is considered that it has been observed that CD95L levels can be elevated in numerous tumors, including breast cancers (*19-23*), and CD95L has been described to be upregulated by tumors of the breast as well (*24*). Elevated levels of CD95L can even serve as a prognostic marker for disease progression and overall survival of patients as

shown for breast cancer (*24-26*). Therefore, the blockage of apoptosis by the mechanism of Bcl-x_L/BAR may have very far reaching implications in light of this alternative CD95 signaling pathway. Since elevated CD95L levels have been observed in breast cancers, this activation may be very relevant, and may require a reevaluation of the current dogma of CD95 signaling as purely apoptotic. Under the model described here, CD95L that is present in the serum of cancer patients, or within the tumors themselves, or upregulated in response to chemotherapy, would result in the induction of non-apoptotic, pro-proliferative and pro-tumorigenic activation that would lead to a faster and more aggressive progression of the disease. This hypothesis is extremely provocative but, I believe is based in enough evidence to warrant investigation. Ultimately, the role of caspase-8 in breast carcinoma cells in both the inhibition of caspase-8 by Bcl-x_L/BAR, and its potential role in the invasive process, may be a therapeutic targets for cells receiving non-apoptotic signaling through CD95. Disruption of this inactivation of caspase-8 and its reactivation may sensitize cells to CD95 mediated apoptosis, and effectively disrupt non-apoptotic CD95 signaling.

KEY RESEARCH ACCOMPLISHMENTS

- Determination of effectiveness of anti-BAR antibody panel
- Determination of immunoprecipitation of BAR using antibody panel
- BAR can be isolated in a complex with caspase-8 using sucrose gradient centrifugation
- MCF7(FB) breast cancer cells activate non-apoptotic pathways following CD95 stimulation
- CD95 stimulation of MCF7(FB) cells results in the activation of a gene program
- CD95 stimulation of MCF7(FB) cells induces *in vitro* invasiveness

REPORTABLE OUTCOMES

Manuscript: "CD95 induces increased motility and invasiveness in apoptosis resistant tumor cells" in revision for EMBO J.

Publications:

Barnhart, B. C., Alappat, E. C., and Peter, M. E. (2003). The CD95 type I/type II model. Semin Immunol *15*, 185-193.

Barnhart, B. C., Lee, J. C., Alappat, E. C., and Peter, M. E. (2003). The death effector domain protein family. Oncogene *22*, 8634-8644.

Barnhart, B. C., and Peter, M. E. (2003). The TNF receptor 1: a split personality complex. Cell *114*, 148-150.

Presentation of poster at: Keystone Symposium on Apoptosis in Development (February, 2004); University of Chicago Committee on Immunology annual retreat (October, 2003); Signal Transduction Symposium, Chicago IL (May 2003).

Award: University of Chicago Cancer Research Foundation Elaine Ehrman Award.

Antibody panel generated: Mouse anti-BAR monoclonal (several hybridoma clones), and rabbit anti-BAR polyclonal antibodies generated.

CONCLUSIONS

The implications of this research are profound. If CD95 is determined to activate non-apoptotic pathways and cause the growth, persistence, or pathogenesis of tumors it would break the dogma of CD95 as a dedicated death receptor. In this field the implications of these observations cannot be understated. Tumor treatment could potentially then be combined with "blocking" CD95 treatment for more effective tumor therapy. Since CD95 is currently considered a death receptor and its apoptotic activity very well established the conclusions generated in this report would force CD95 to be viewed in a vastly different light. Because of the profound implications of these observations and because they fall within the scope of the original statement of work in "The role of caspase-8 in breast carcinoma cells", I have performed many experiments on these observations in the preceding year, as well as the characterization of the anti-BAR antibody panel and the sucrose gradient experiments. The studies have generated many very striking data and the manuscript that has arisen from these investigations is attached. The pursuit of these observations has been very rewarding, and continues to be so. Because they still fall within the scope of the original proposal as they directly address the role of caspase-8 in breast carcinoma cells (indeed MCF7(FB) cells were the primary model used in these studies), I performed the experiments described.

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CD95 engagement increases motility and invasiveness of apoptosis resistant tumor cells through activation of a combination of different signaling pathways

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Summary

The apoptosis inducing death receptor CD95 (APO-1/Fas) controls the homeostasis of many tissues such as lymphoid tissues. Despite its apoptotic potential most human tumors are refractory to the cytotoxic effects of CD95 ligand. This is due either to upregulation of antiapoptotic genes, functional elimination of CD95 apoptosis signaling pathway components, mutation or downregulation of CD95. Here we show that CD95 stimulation of apoptosis resistant tumor cells by either membrane bound or soluble CD95 ligand specifically induces increased motility and invasiveness, a response much less efficiently triggered by TNFα or TRAIL. Engagement of CD95 triggered activation of at least 5 different signaling pathways 3 of which - activation of NF-κB, Erk1/2 and caspase-8 - were found to be important to this novel activity of CD95. A gene chip analysis suggested that the combination of these pathways ultimately dictates transcriptional activation of genes that promote survival, cell proliferation and invasiveness suggesting that CD95 apoptosis resistant cells receive a selective growth advantage when stimulated by CD95 ligand. Elevated CD95 ligand levels can be found in many human cancer patients either systemically in the serum or within the tumor itself, which has mainly been viewed as an immune escape mechanism. Our data suggest that such CD95L could have tumorigenic activities on human cancer cells. This could become highly relevant during chemotherapy which can cause upregulation of CD95L by both tumor and nontumor cells.

Introduction

CD95 ligand (CD95L/FasL), a member of the death ligand family, that also includes TNFα and TRAIL has mainly been viewed as an apoptosis inducing ligand and members of this family are therefore being considered as potential antitumor reagents (Gardnerova *et al.,* 2000). CD95L is expressed either on cell membranes (mCD95L) or in a soluble form (sCD95L) and induces apoptosis when it triggers its cognate receptor CD95 (APO-1/Fas) (Peter *et al.,* 2003). Upon binding of CD95L (or agonistic antibodies), CD95 assembles the death inducing signaling complex (DISC) comprised of CD95, the adaptor molecule FADD, procaspase-8, procaspase-10 and the caspase-8/10 regulator c-$FLIP_L$ (Peter and Krammer, 2003). We have previously demonstrated that cells can die in different ways following CD95 activation, either dependent on (Type II) or independent of (Type I) mitochondria (Scaffidi *et al.,* 1998; Barnhart *et al.,* 2003). We recently found that Type I cells correspond to mesenchymal tumors whereas Type II cells have a more epithelial phenotype (Algeciras-Schimnich *et al.,* 2003). sCD95L was only cytotoxic to Type II/mesenchymal tumor cells and did not kill Type I/epithelial cells.

Since chemotherapeutic drugs can cause upregulation of CD95L it is believed that this contributes to the elimination of tumor cells by inducing their apoptosis (Friesen *et al.,* 1996). However, many tumor cells are resistant to CD95 mediated apoptosis, especially after therapy (Friesen, *et al.,* 1999). We recently determined that about two thirds of the 60

tumor cell lines of the NCI drug screening panel (NCI60) are completely resistant to CD95 mediated apoptosis with little correlation between CD95 apoptosis sensitivity and CD95 surface expression (Algeciras-Schimnich *et al.,* 2003). Although it has been recognized that CD95 stimulation of certain cells under certain conditions can cause induction of genes that have functions outside of apoptosis (Faouzi *et al.,* 2001; Park *et al.,* 2003) and that CD95 can promote proliferation of T cells (Alderson *et al.,* 1993; Alam *et al.,* 1999; Kennedy *et al.,* 1999) specific tumor promoting effects of CD95 stimulation for CD95 apoptosis resistant tumor cells have not been reported.

Recently, a first report linked stimulation of CD95 with increased growth of tumor cells *in vivo* (Lee *et al.,* 2003). A lung carcinoma cell line stably expressing CD95 showed accelerated growth in syngeneic wild type mice when compared to *gld* mice which lack expression of functional CD95L. However, since these cells were CD95 apoptosis sensitive *in vitro* it could not be demonstrated that direct stimulation of CD95 on these tumor cells was responsible for these effects and the nature of the pathway(s) triggered though CD95 could not be studied.

We now have performed an analysis of the responses of a panel of CD95 apoptosis resistant tumor cell lines by stimulating them with sCD95L, mCD95L or agonistic anti-CD95 antibodies. Our data demonstrate that a significant number of these cells respond to any stimulation of CD95 with increased motility and invasiveness through Matrigel coated

membranes. We determined that stimulation of CD95 induces at least 5 different apoptosis independent pathways of which at least three (activation of caspase-8, NF-κB and Erk1/2) are independently required to different extents for the increased motility and invasiveness observed with these tumor cells. The effect is selective for CD95 stimulation since treatment of apoptosis resistant cells with either TNFα or TRAIL only marginally induced motility or invasiveness. Since both of the latter cytokines are also efficient activators of NF-κB these observations suggest that the nonapoptotic response of tumor cells to stimulation of CD95 requires activation of multiple signaling pathways. Our data suggest that elevated levels of CD95L found in cancer patients could contribute to increase tumorigenicity of tumor cells.

Results

Stimulation of CD95 on CD95 apoptosis resistant tumor cells induces increased invasiveness

It has been previously suggested that soluble CD95L (sCD95L) is very inefficient in inducing apoptosis in tumor cells (Schneider *et al.,* 1998; Tanaka *et al.,* 1998) and although it was shown that sCD95L can activate other nonapoptotic pathways (Ahn *et al.,* 2001) the range of biological responses of tumors cells to this stimulus are unknown. The same is true for apoptosis resistant tumor cells that occur either spontaneously or are selected for by chemotherapy (Friesen *et al.,* 1999). Additionally, it is well established that as part of carcinogenesis tumors often either inactivate the apoptosis inducing activity of CD95 by downregulating or inactivating CD95 or its proapoptotic signaling molecules or by upregulation of antiapoptotic proteins such as Bcl-2 or c-FLIP (Peter *et al.,* 2003). Since increased expression of sCD95L is frequently found in cancer patients and is associated with various forms of cancer (Owen-Schaub *et al.,* 2000) we asked whether stimulation of CD95 on CD95 apoptosis resistant tumor cells provokes protumorigenic responses.

To directly address this question we performed *in vitro* invasiveness assays (Figure 1). In this assay tumor cells were placed in the top of two chambers separated by a Matrigel coated membrane (pore size 8 μm) in serum free medium. The bottom chamber contained 10% FCS. The anti-CD95 stimulus (either anti-CD95 antibody or CD95 ligand) was added

to both the top and bottom chamber. Any response by tumor cells in this assays was therefore not caused by a chemoattraction of tumor cells toward CD95L - an activity previously reported for neutrophils and phagocytes (Ottonello *et al.,* 1999; Seino *et al.,* 1998) - but was the result of stimulation of surface CD95 on the tumor cell ultimately triggering a program of increased motility and invasiveness though the basement membrane mimetic Matrigel.

To test the effects of triggering CD95 on apoptosis resistant tumor cells under controlled conditions we first tested MCF7-Fas-Bcl-x_L(FB) breast carcinoma cells which express high levels of CD95 (Algeciras-Schimnich *et al.,* 2003) and are rendered apoptosis resistant by the anti-apoptotic protein Bcl-x_L (Stegh *et al.,* 2002). These cells are a model for a tumor cell that has acquired apoptosis resistance through upregulation of a Bcl-2 family member (which is frequently found in human tumors). As expected in apoptosis sensitive MCF7-Fas control cells CD95 stimulation did not promote invasiveness (Figure 1A) but induced apoptosis (data not shown). Unstimulated MCF7(FB) cells also exhibited low invasiveness. However, when these cells were exposed to either the agonistic anti-CD95 mAb anti-APO-1, leucine zipper tagged CD95L (LzCD95L) or sCD95L they migrated through Matrigel. This response was not limited to MCF7(FB) cells since it was also observed in a naturally CD95 resistant ovarian tumor cell line SK-OV-3. Again all three CD95 specific stimuli induced increased invasiveness of SK-OV-3 cells (Figure 1A).

Furthermore, 14 other cell lines showed a clear increase of invasiveness when stimulated through CD95 (Figure 1B and Figure 6C).

In vivo tumor cells most likely encounter CD95L in two different forms: sCD95L in the serum and membrane bound (m)CD95L e.g. on tumor infiltrating lymphocytes, proinflammatory cells or stromal cells. This could potentially involve cell-cell interaction between tumor cells and CD95L expressing cells. To test whether cell bound human mCD95L could trigger increase in motility and/or invasiveness of CD95 apoptosis resistant tumors we incubated MCF7(FB) cells with a cell line expressing authentic unmodified human mCD95L (CT26L, Aoki *et al.,* 2001). When mixed at a ratio of 3.3:1 CT26L cells induced maximal motility (as determined in a migration assay, without Matrigel) of MCF7(FB) cells (Figure 1C) although significant increase of motility was observed when mixed at a ratio of 1:1 or 6.7:1. The activity of the CT26L cells was due to the expressed human mCD95L since it could be significantly inhibited by a neutralizing anti-human CD95L mAb NOK-1. CT26L cells also induced invasiveness which was not observed with the parental CT26 cells that do not express human CD95L (Figure 1C). In summary, any tested form of stimulation of CD95 including sCD95L and physiological unmodified mCD95L can induce increase in motility and invasiveness of CD95 resistant tumor cells.

To directly test the form of CD95L tumor cells might encounter *in vivo* we incubated MCF7(FB) cells with human peripheral blood mononuclear cells (PBMC) (Figure 1D).

Resting PBMCs did not show any effect on the invasiveness of the tumor cells. However, when the tumor cells were mixed with PBMCs that had been activated with phytohemagglutinin (24 hrs) followed by stimulation with interleukin 2 for 5 days, which selectively activates T lymphocytes, an increase in invasiveness could be detected (Figure 1D). This activity of the activated PBMCs was primarily due to CD95L since it could be substantially be blocked by the CD95L neutralizing antibody.

CD95 stimulation of CD95 apoptosis resistant MCF7(FB) cells results in transcriptional upregulation of anti-apoptotic, proproliferative and potentially protumorigenic genes

To test whether triggering of CD95 induced transcriptional upregulation of genes that would be consistent with a protumorigenic nature of the response observed with apoptosis resistant tumor cells we treated MCF7(FB) cells for 8 hrs with anti-APO-1 and subjected their mRNA to a gene array analysis using the Affymetrix U133 gene chip set covering 33,000 distinct human genes (Figure 2A and suppl. Figure S1). Of these genes, 121 (0.0037%) were induced greater than 2 fold and the induction of 8 of these genes was confirmed by real-time PCR (Figure 2B). These genes were also induced when cells were triggered with LzCD95L (data not shown). Upregulated genes were functionally grouped into antiapoptotic, proproliferative and protumorigenic genes (Figure 2A). Interestingly, 16 of these genes were known targets of the transcription factor NF-κB. It has been

demonstrated that the NF-κB pathway can block apoptosis by death receptors as well as promote tumor progression (Orlowski and Baldwin, 2002) and NF-κB has been shown to be activated in cells after triggering of CD95 (Ponton *et al.,* 1996; Wajant *et al.,* 2000; Packham *et al.,* 1997). However, these studies focused on only a few cell lines and the functional relevance of this activation remained unknown. Our data now suggested that activation of NF-κB may be involved in the increased invasiveness of CD95 stimulated tumor cells.

The majority of CD95 apoptosis resistant tumor cells respond to anti-CD95 stimulation with activation of NF-κB

To test whether activation of NF-κB could be involved in the invasiveness of CD95 stimulated tumor cells we first tested MCF7(FB) cells for CD95 induced NF-κB activation. Electromobility shift assays (EMSA) confirmed activation of NF-κB in these cells when stimulated through CD95 by both the agonistic anti-CD95 antibody anti-APO-1 and two CD95 ligand preparations (Figure 3A). Activation of NF-κB was first detectable 1 hour after CD95 stimulation and it progressively increased reaching levels almost as high as those induced by TNFα (Figure 3A). Activation of NF-κB was likely a direct effect as CD95 induced activation of NF-κB was not prevented by cycloheximide pre-treatment

(Figure S2A). Supershift analysis identified the canonical p50/p65 heterodimer as the predominant complex activated by CD95 (Figure S2B).

To assess the significance of the activation of NF-κB in CD95 resistant tumor cells we monitored NF-κB activation in cell lines of the anti-tumor drug screening panel of the NCI (NCI60). We previously identified the majority of these cell lines to be resistant to CD95-mediated apoptosis, many of which expressed high levels of surface CD95, suggesting that they developed mechanisms to withstand apoptotic CD95 stimulation (Algeciras-Schimnich *et al.,* 2003 and data not shown). 13 of these cell lines were selected randomly and examined for their ability to activate NF-κB upon CD95 triggering. Most cell lines (11/13, 85%) activated NF-κB in response to one or more of the CD95 specific stimuli with only two lines exhibiting no CD95 induced NF-κB complexes (supplementary Figure S3A).

Our recent analysis of the NCI60 cells demonstrated that Type I but not Type II cells (both of which are apoptosis sensitive to crosslinked agonistic anti-CD95 antibodies or highly aggregated CD95L) are resistant to the toxic effects of soluble CD95L (sCD95L) (Algeciras-Schimnich *et al.,* 2003). We now demonstrate that sCD95L induces NF-κB activation in the prototype Type I tumor cell lines SKW6.4 and H9 (supplementary Figure S3B), and in 7 of 8 cell lines randomly picked from the 11 NCI60 we identified as Type I cells (supplementary Figure S3C). In summary, the majority of all CD95 apoptosis resistant cell lines tested, and even of the CD95 sensitive Type I cell lines stimulated with sCD95L,

from a variety of histological origins responded to CD95 stimulation with activation of the NF-κB pathway, indicating that CD95L induced NF-κB activation is widely found in tumor cells.

CD95-induced invasiveness requires activation of NF-κB

We next tested whether the CD95-induced invasiveness was indeed dependent on activation of NF-κB. Incubation of MCF7(FB) cells with a cell permeable GST fusion protein of a non-degradable IκBα mutant protein (GST-TAT-IκBα^{mut}) efficiently suppressed activation of NF-κB as evidenced by inhibition of NF-κB DNA binding (Figure 3B) and it blocked CD95 induced invasiveness (Figure 3C). A migration assay demonstrated that the effect of NF-κB was in part due to increased motility (Figure 3D).

CD95L is more potent in inducing invasiveness than TNFα or TRAIL

The data so far suggested that activation of NF-κB was involved in CD95 induced motility and invasiveness. CD95 triggering resulted in activation of the canonical NF-κB p50/p65 heterodimer that is also activated by treating cells with TNFα (Figure 3A and Tang *et al.,* 2001). However, in both the EMSA analysis as well as by measuring increase in transcriptional activity using a κB driven CAT reporter gene assay TNFα was more active in inducing NF-κB (Figure 4A) without causing any signs of toxicity (as monitored

by MTS assays, data not shown). We therefore tested the activity of TNFα and also the CD95L related death ligand TRAIL to induce invasiveness of MCF7(FB) cells (Figure 4B). TNFα and TRAIL only marginally induced invasiveness in these cells. When compared side by side both TNFα and LzCD95L induced activation of the IκB kinase complex as evidenced by activation of its subunit IKKβ (Figure 4C) and degradation of IκBα (data not shown). The activation of NF-κB through CD95 was somewhat delayed compared to TNFα induced NF-κB activation suggesting a different pathway upstream of the IKK complex. Upstream of the IκB kinases we identified NEMO to be an essential component in the pathway of both death receptors since a peptide that blocks interaction of NEMO with the IKK complex (May *et al.,* 2000) efficiently prevented activation of NF-κB as evidenced by both EMSA (Figure S2C) and an ELISA based assay that detects activated p65 (Figure S2D). We therefore tested the role of the receptor proximal component of the TNF pathway, RIP in CD95 mediated activation of NF-κB by testing RIP deficient Jurkat cells. RIP was required for TNF induced activation of NF-κB but dispensable for CD95 induced NF-κB activation (Figure 4D). In summary, our data indicate that NF-κB activation is required but not sufficient for tumor cells to demonstrate increased motility and invasiveness suggesting involvement of other pathways that either emanate in the pathway somewhere upstream of the IKK complex or that act in parallel to the NF-κB pathway.

Requirement of the Erk signaling pathway for CD95 induced invasiveness

CD95 activates the Erk MAP kinase pathway and this activation has been shown to be critical for proproliferative effects of CD95 during neurite outgrowth of certain neurons (Desbarats *et al.,* 2003). The other two major MAP kinase pathways p38 and JNK have also been shown to be activated by CD95 under certain conditions (Toyoshima *et al.,* 1997). Western blot analyses using pairs of anti-phospho site directed antibodies and their corresponding panspecific antibodies against the three major kinases, Erk1/2, JNK1/2 and p38 determined that all three MAP kinases are activated upon stimulation of CD95 in MCF7(FB) cells (Figure 5A). The p38 inhibitor SB203580 had no effect on invasiveness (while it completely abolished activation of p38, data not shown) excluding a role of p38 in the CD95 induced invasiveness. A contribution of JNK kinases to the *in vitro* invasiveness could not be determined since inhibition of JNK reduced viability of these cells (as assessed by a MTS assay, not shown) thereby interfering with the invasiveness. Treatment of MCF7(FB) cells with LzCD95L induced activation of the Erk1/2 MAP kinases which was completely prevented by the MEK1/2 inhibitor PD98059 (Figure 5B). Interestingly, addition of caspase inhibitors did not inhibit Erk activation suggesting that these events occur independently. This conclusion is consistent with the finding that activation of Erk through CD95 is independent of its death domain (DD) (Desbarats *et al.,* 2003). When we treated MCF7(FB) cells with the NEMO inhibiting peptide, which resulted in a profound

inhibition of NF-κB activation in CD95 stimulated cells (Figure 5C, see also Figure S2C), no effect was seen on activation of Erk indicating that CD95 induced activation of Erk is also independent of activation of NF-κB. To determine the functional consequences of Erk activation we tested the effects of the PD inhibitor in the *in vitro* invasiveness assay (Figure 5D). PD98059 treatment significantly inhibited invasiveness. This result indicates that like activation of NF-κB activation Erk contributes to CD95 induced invasiveness. To test whether the MAP kinase pathway acts independently or in conjunction with NF-κB, we determined the effects of PD98059 on a NF-κB driven CAT reporter assay. The PD inhibitor caused only a minor reduction of reporter gene activity (Figure 5E).

Involvement of active caspase-8 in CD95 induced invasiveness

We recently demonstrated that MCF7(FB) cells process caspase-8 despite their resistance to CD95 mediated apoptosis (Stegh *et al.,* 2002). Active caspase-8 could therefore play a role in the increased invasiveness observed upon CD95 stimulation. Treatment of these cells with the oligo specific caspase inhibitor zVAD-fmk or the caspase-8 selective inhibitor zIETD-fmk completely blocked CD95 induced invasiveness in MCF7(FB) cells (Figure 6A). Inhibition of caspases had no effect on CD95 induced increase in NF-κB DNA binding (data not shown). Furthermore, it also did not prevent NF-

κB-dependent transcriptional activation (Figure 6B) suggesting that caspase-8 acts downstream of the transcriptional activation induced by activated NF-κB.

To determine the contribution of both the MAP kinase pathway and caspase-8 activation to the increase in invasiveness in untransfected tumor cells we pretreated 6 of the NCI60 cells with either the PD inhibitor or zIETD-fmk followed by stimulation with sCD95L (Figure 6C). In all cases we detected increased invasiveness which was blocked by inhibition of MAP kinases. In contrast, inhibition of caspase-8 significantly inhibited invasiveness of most but not all cells. Whereas invasiveness of EKVX and TK-10 cells was critically dependent on activation of caspase-8 in untransfected MCF7 cells caspase-8 activation did not play a significant role in this process. In summary, multiple pathways are activated and least three of them, activation of caspase-8, NF-κB and Erk1/2 independently contribute to the response of apoptosis resistant tumor cells to CD95 stimulation to varying extents with increased motility and invasiveness, indicating a high degree of complexity of regulation of this process.

Discussion

In this report we have demonstrated that stimulation of CD95 apoptosis resistant tumor cells by any form of CD95L triggers signaling pathways that can increase their motility and invasiveness. This activity of CD95 is not limited to certain forms of cancers since tumor cells representing malignancies from ovary (OVCAR-4, SK-OV-3), breast (MCF7), skin (MALME-3M, SK-MEL-5, UACC-62, UACC-257), lung (A549, EKVX, HOP-62, NCI-H522), and kidney (ACHN, CAKI-1, TK-10. UO-31) all showed a similar responses to CD95 stimulation. CD95 ligand has not been previously reported to have this effect on tumor cells. The only report linking CD95 with migrational activity came from an analysis of renal tubular epithelial cells treated with agonistic anti-CD95 antibodies (Jarad *et al.*, 2002). However, this form of migration was shown to depend on CD95 induced expression of $\alpha_v\beta_8$ integrin (which was not found to be induced in MCF7 cells, also used in our study) and correlated with migration to vitronectin.

We determined that at least three pathways can be activated by CD95 to elicit the nonapoptotic responses in tumor cells: activation of caspase-8, Erk1/2 and NF-κB. These pathways regulate gene expression, increased invasiveness and increased motility. We found that caspase-8 activity is involved in the increased motility of certain tumor cells, inhibition of caspase-8 completely blocked CD95 induced motility without interfering with the activation of NF-κB or any of the MAP kinase pathways, Erk1/2 (Figure 6A), JNK or

p38 (data not shown) in MCF7(FB) cells. Caspase-8 has not been implicated in CD95 induced motility. The only report that has associated caspases with this type of cellular response described the requirement of an unidentified caspase(s) in the spreading activity of cells on collagen and poly-L-lysine coated plates (Watanabe and Akaike, 1999).

Activation of Erk1/2 through CD95 has recently been shown to be required for neurite outgrowth of certain neurons (Desbarats *et al.,* 2003) and we now demonstrate that inhibition of this pathway substantially inhibits CD95 induced invasiveness. Interestingly, like caspase-8, inhibition of Erk1/2 did not interfere with activation of NF-κB, the third pathway triggered by CD95 that is important for CD95 induced invasiveness, nor did inhibition of NF-κB have any effect on activation of Erk. The data presented therefore indicate that these three major pathways act in parallel. Consistent with the role of NF-κB in tumor development (Lin and Karin, 2003) a gene chip analysis suggested a protumorigenic activity of NF-κB through transcriptional activation of genes. However, like the other pathways, activation of NF-κB was found to be required yet not sufficient to mediate the nonapoptotic effects of CD95.

Our data suggest that CD95 activates NF-κB through the canonical pathway of activation of the IKK complex (Figure 4C and S2C and D) and degradation of IκBα (not shown). It was therefore surprising that the related death ligand TNFα, which activates NF-κB more efficiently than CD95L, and TRAIL (which also induced NF-κB activation in

MCF7(FB) cells, Figure 3A) induced invasiveness in CD95 apoptosis resistant cells much less efficiently than CD95L. This result is consistent with a model in which multiple signaling pathways must be activated in parallel, and not NF-κB activation alone, to induce the maximal effect and could be due to the differences in upstream signaling events that link the death receptors to the IKK complex. CD95 induced NF-κB activation was recently shown to require both NIK and IKKβ (Russo *et al.,* 2002). In another study it was shown that overexpression of the CD95 DISC components, caspase-8, 10 and c-FLIP results in activation of the IKK complex through activation of NIK and requires the protein RIP suggesting that CD95, similar to TNF receptor I, involves RIP for activation of NF-κB (Shikama *et al.,* 2003). In contrast to this prediction our study, while confirming that TNFα induced activation of NF-κB critically dependents on RIP (Kelliher *et al.,* 1998; Pimentel-Muinos and Seed, 1999), demonstrates that RIP, at least in Jurkat T cells, is not required to activate NF-κB when CD95 is stimulated by either CD95L or agonistic anti-CD95 Ab when compared to overexpressing its signaling components. This is consistent with recent data that suggest that stimulation of CD95 with a nonfunctional DD still results in activation of NF-κB (BC Barnhart, P Legembre, ME Peter, unpublished data and Ponton *et al.,* 1996) indicating that the DISC may not be required for the activation of this pathway.

The CD95 apoptosis resistance by the tumor cells in our study likely represents a multitude of different mechanisms. Consistent with its proapoptotic function tumors can acquire resistance to CD95 stimulation by upregulation of antiapoptotic Bcl-2 family members (Scaffidi *et al.,* 1998), upregulation of the caspase-8 protease inactive homologue

c-FLIP$_L$ (Tschopp *et al.,* 1998), or by the silencing or deleting the caspase-8 gene (Teitz *et al.,* 2000; Mandruzzato *et al.,* 1997). CD95 apoptosis resistance may also be dependent on mutations that render CD95 inactive or on the downregulation of CD95 expression (Reichmann, 2002; Müschen *et al.,* 2002b; Bullani et al. 2002). CD95 is primarily viewed as an apoptosis-inducing receptor. However, if this were the sole function of the receptor one would expect to find tumors that either delete the CD95 gene or completely block its expression by promotor hypermethylation. However, a complete loss of CD95 expression has not been reported, suggesting that tumors derive an advantage from maintaining expression of CD95.

CD95 has previously been demonstrated to trigger proproliferative signaling pathways especially in peripheral T cells (Alderson *et al.,* 1993; Alam *et al.,* 1999; Kennedy *et al.,* 1999). However, the consequences of such stimulation for tumor cells were not known. Recently, it was found that reconstitution of a caspase-8 deficient human neuroblastoma cell line with procaspase-8 greatly increased its ability to form tumors in nude mice, suggesting that similar to CD95 some of the essential apoptosis signaling components of the DISC have dual functions that can promote tumor growth (Teitz T, Grenet J, Lahti JM and Kidd VJ, unpublished observations).

Our data provide the mechanistic basis for a recent report that demonstrated such a tumor promoting function of CD95. Increased growth of a lung carcinoma cell line stably expressing CD95 was observed *in vivo* in syngeneic mice when compared to *gld* mice

lacking expression of functional CD95L (Lee *et al.,* 2003). The tumor promoting activity of CD95 required the CD95 intracellular domain and therefore likely involved CD95 signaling pathways. Interestingly, no further CD95 stimulation was involved beyond that naturally occurring in the mice, consistent with the idea that tumor cells *in vivo* are likely exposed to CD95L that is expressed on tumor infiltrating lymphocytes or in the serum. In a preliminary experiment we demonstrated that CD95L produced by activated human PBMCs (mostly T-cells) can induce invasiveness of apoptosis resistant tumor cells.

Systemically increased CD95L levels have frequently been reported to be associated with the following human cancers: bladder carcinoma (Mizutani *et al.,* 2001; Mizutani *et al.,* 2002), breast cancer (Müllauer *et al.,* 2000; Müschen *et al.,* 1999; Gutierrez *et al.,* 1999; O'Connell *et al.,* 1999; Müschen *et al.,* 2000), pancreatic carcinoma (Bellone *et al.,* 2000), testicular germ cell tumors (Hara *et al.,* 2001), gastric carcinoma (Ichikura *et al.,* 2001), hepatocellular carcinoma (Nakamoto *et al.,* 1999), nasal lymphoma (Sato *et al.,* 1996), large granular lymphocytic leukemia (Saitoh *et al.,* 2000; Tanaka *et al.,* 1996), natural killer cell lymphoma (Murayama *et al.,* 1999; Tani *et al.,* 1999; Tanaka *et al.,* 1996), acute lymphocytic lymphoma, adult T cell leukemia, cutaneous T cells lymphoma (Takubo *et al.,* 2000), and B cell non-Hodgkin's lymphoma (Kanda *et al.,* 1999). In addition, direct upregulation of CD95L in the tumor itself has also been shown for colon cancer (Song *et al.,* 2001), testicular germ cell tumors (Hara *et al.,* 2001) and breast cancer

(Reimer *et al.,* 2000). Elevated levels of CD95L can even serve as prognostic markers for disease progression and overall survival of patients as shown for bladder cancer (Mizutani *et al.,* 2002); gastric carcinoma (Tsutsumi *et al.,* 2000), and breast cancer (Bewick *et al.,* 2001; Mottolese *et al.,* 2000; Reimer *et al.,* 2000).

Elevated levels of CD95L produced by tumor or nontumor cells in cancer patients are currently thought to promote tumor growth in two ways: 1. Upregulation of CD95L by tumor cells has been implicated in killing attacking cytotoxic T lymphocytes (CTLs) resulting in specific immune suppression. This has been referred to as the "tumor strikes back" theory (Walker *et al.,* 1997). However, in many cases this theory could not be confirmed, suggesting that tumors may benefit from elevated CD95L in ways other than through facilitating immune escape (Green and Ferguson, 2001). Furthermore, the systemic increase of soluble CD95L in the serum of many cancer patients would not be consistent with a specific immune suppression. 2. Increased levels of sCD95L, in the serum of cancer patients has been suggested to antagonize the cytotoxic effects of membrane bound CD95L that could be used by CTLs to eliminate tumor cells (Suda *et al.,* 1997; Mizutani *et al.,* 2001). This model is based on reports that demonstrated that sCD95L is orders of magnitude less active than mCD95L (Schneider *et al.,* 1998; Tanaka *et al.,* 1998). However, we recently demonstrated that sCD95L is cytotoxic to Type II/mesenchymal tumor cells (Algeciras-Schimnich *et al.,* 2003) and only Type I/epithelial cells are resistant

to the toxic effects of sCD95L. We also demonstrated that Type I cells are much less sensitive to the apoptosis inducing activities of mCD95L. An important question which has not been addressed is therefore whether CD95L has other activities on both tumor cells that are either completely apoptosis resistant or on Type I tumor cells (which are viewed as the most CD95 apoptosis sensitive cells since they are highly apoptosis sensitive to artificially aggregated CD95L or agonistic anti-CD95 Abs but in fact are virtually resistant to the effects of physiological sCD95L and have low sensitivity to mCD95L (Algeciras-Schimnich *et al.,* 2003)). We have now demonstrated that two of the most CD95 positive Type I tumor cell lines, the H9 T cell leukemic cells and the B lymphoblastoid cell line SKW6.4 respond to stimulation by sCD95L with activation of NF-κB and identified 4 CD95 sensitive Type I cells (ACHN, CAKI-1, TK-10, UO-31; interestingly all representing renal carcinomas) to respond to sCD95L with increased invasiveness (see Figure 1B and Figure 6C).

Our data demonstrate for the first time that both soluble and membrane bound CD95L elicit biological effects on multiple CD95 apoptosis resistant tumor cells and suggest that tumors gain a direct advantage from an increase in CD95L levels through triggering multiple apoptosis independent pathways resulting in upregulation of proproliferative/antiapoptotic and tumorigenic genes and/or an increase in motility and invasiveness. In humans, elevated levels of sCD95L have also been reported in cancer patients in response to chemotherapy (Bewick *et al.,* 2001; Kanda *et al.,* 1999) and widely

used chemotherapeutic drugs such as adriamycin or 5-fluorouracil have been shown to cause secretion of active sCD95L by human tumor cells *in vitro* (Friesen *et al.,* 1996) and a systemic increase of CD95L in mice *in vivo* (Eichhorst *et al.,* 2001), respectively. Future experiments will investigate the possible tumorigenic activity of CD95L that is produced in response to exposure to chemotherapeutic drugs for CD95 apoptosis resistant tumors. Our study could therefore affect the way tumor therapy is currently performed.

Materials and methods

Cells and reagents and general procedures

MCF7-Fas vector (MCF7(FV)) and MCF7-Fas-Bcl-x_L (MCF7(FB)) were cultured as described (Stegh *et al.,* 2002). All other cell lines were cultured as described previously (Algeciras-Schimnich *et al.,* 2003). The anti-CD95 mAb anti-APO-1 has been described previously (Scaffidi *et al.,* 1999). The MAP-K inhibitors PD98059, SB203580 and the caspase inhibitors, zIETD-fmk and zVAD-fmk, were purchased from Calbiochem (San Diego, CA). All other chemicals used were of analytical grade and purchased from Sigma or Molecular Probes. The plasmid to produce the recombinant shCD95L S2 (Tanaka *et al.,* 1995), was provided by Dr. Shigekazu Nagata (Osaka Bioscience Institute, Osaka, Japan). Generation of sCD95L and LzCD95L and apoptosis assays were performed as described previously (Algeciras-Schimnich *et al.,* 2003). TNFα was purchased from Peprotech and used at a concentration of 1000 U/ml. When inhibitors were used cells were pre-incubated with for 30 min (zVAD-fmk, zIETD-fmk, PD98059, SB203580) or pre-incubated 2 hours (GST-TAT-IκBα^{mut} or GST-TAT) and then incubated for 16 hrs with sCD95L or anti-APO-1 in 96-well plates. All inhibitors used were tested for toxicity on all cells used by performing a MTS assay as described (Algeciras-Schimnich *et al.,* 2003) after a 3 day incubation.

In vitro invasiveness and motility assay

Biocoat Matrigel invasion chambers (BD Biosciences) containing 8μm pore size positron emission tomograph membranes were cultured in 24-well plates. After rehydration of the Matrigel, 75,000 cells were added to the top chamber in serum free media. The bottom chamber was filled with serum containing media. Stimulus (anti-APO-1 antibody or recombinant soluble CD95 ligand, TNFα, or recombinant LzTRAIL) or control supernatants were added to both chambers of each well. Cells were cultured for 72 hours at 37°C in a 5% CO_2, humidified incubator. To quantify invasion, cells were removed from the top side of the membrane mechanically using a cotton tipped swab and invading cells were fixed with methanol and stained with Giemsa stain and five representative fields were counted for each insert. For experiments in which cells were incubated with inhibitors, cells were plated with inhibitors for two hours prior to addition of stimulus. GST-TAT-IκBα^{mut} and GST-TAT was added each day at 10 μM. CT26 cells were fixed for 5 min in 2% paraformaldehyde and washed extensively prior to addition to wells. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats purchased from Life Source (Glenview, IL). Erythrocytes were removed by centrifugation on a gradient of lymphocyte separation media (BioWhittaker). Resulting PBMC were then washed and incubated for 16 hours with 2 μg/ml phytohemagglutinin(PHA)-L (Sigma) followed by six days with 25 U/ml recombinant IL-2 (Sigma). Resting cells were maintained in RPMI with 10% FBS. For antibody blocking experiments, stimulator cells were pre-incubated with NOK-1

(Pharmingen) for 45 min and cultures were maintained with 10 μg/ml NOK-1 for the duration of the experiment. For migration assays Transwell inserts chambers with 8 μm pores (Costar) were used.

Western Blotting

Cells were lysed in lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1mM PMSF, and protease inhibitor cocktail (Sigma, St. Louis, MO)) and protein concentration determined by Bradford assay (Biorad Laboratories). Lysates (100 μg) were resolved on 12% acrylamide gels and blotted with the indicated antibodies. IKKβ was purchased from Santa Cruz and β-actin antibody from Sigma. Antibodies for MAPK proteins (ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK) were from Cell Signaling Technology.

In vitro kinase assay

MCF7(FB) cells were incubated with stimulus (1μg/ml LzCD95L or 1000U/ml TNFα) for the indicated time. *In vitro* kinase assays were performed as described previously (Mercurio *et al.,* 1997). Briefly, cells were washed and lysed in lysis buffer (as above) containing 300 mM NaCl. Immunoprecipitation of 200μg of protein was performed for 16 hours at 4°C

with anti-IKKβ antibodies. Immunoprecipitates were incubated with 2μg of GST-IκBα for 30 min at 30°C in the presence of 1μCi of ^{32}P-γATP in kinase buffer (Mercurio *et al.*, 1997). The reaction was then resolved on a 12% polyacrylamide gel, dried and exposed to film. Western blots were performed as control and blotted with antibodies for IKKβ and β-actin.

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Figure legends

Fig. 1. Stimulation of CD95 in CD95 apoptosis resistant tumor cells induces invasiveness. (**A**) 3 day *in vitro* invasiveness assay of MCF7(FB) and SK-OV-3 cells incubated with anti-APO-1 (αA), LzCD95L (Lz) or sCD95L (S). Inset shows fixed and stained cells which migrated through a Matrigel-coated membrane unstimulated (-) or stimulated (+) with anti-APO-1. (**B**) Invasiveness assay of apoptosis resistant cells (left panel) following stimulation with anti-APO-1 (asterisk), LzCD95L (underlined) or sCD95L. (**C**) Invasiveness (left) and motility (right) assay of MCF7(FB) cells incubated with anti-APO-1 (αA), CT26L or CT26 cells at the ratios 1:1, 3.3:1 (labeled as 3:1) and 6.7:1 (labeled as 7:1). The ratio of CT26 to MCF7(FB) cells in the invasiveness assays was 6.7:1. (**D**) PBMCs which had been activated for 16 hours with PHA, followed by six days with IL-2 (A) or were not stimulated with either PHA or IL-2 (R) were cocultured with MCF7(FB) cells at a 6.7:1 ratio in an invasiveness assay. Where indicated the PBMCs were preincubated with NOK-1. NOK-1 was maintained in the culture at 10μg/ml.

Fig. 2. CD95 induced gene activation in CD95 apoptosis resistant MCF7(FB) cells. (**A**) MCF7(FB) cells were stimulated with anti-APO-1 (8 hrs) and mRNA was subjected to gene chip analysis. Genes were placed in five groups according to function. Numbers in parentheses correspond to fold induction. Genes in bold are known NF-κB targets. (**B**)

Validation of gene screen by real-time PCR of a number of genes induced in MCF7(FB) cells stimulated for 8 hours with anti-APO-1.

Fig. 3. CD95 mediated activation of NF-κB contributes to *in vitro* invasiveness. (**A**) EMSA analysis of NF-κB activation of CD95 stimulated MCF7(FB) cells or cells treated with TNFα or LzTRAIL. (**B**) EMSA analysis of NF-κB activity of cell extracts of MCF7(FB) cells stimulated with LzCD95L for 4 hours and after pretreatment with either GST-TAT-IκBα^{mut} or GST-TAT control (tat). (**C**) Invasiveness assay and (**D**) migration assay of MCF7(FB) cells incubated with GST-TAT-IκBα^{mut} (mut) or GST-TAT control (t).

Fig. 4. CD95L is more efficient than TNFα or TRAIL in inducing increased motility and invasiveness of MCF7(FB) cells. (**A**) CAT reporter gene analysis of MCF7(FB) cells transfected with a NF-κB promoter (κB) or mutated NF-κB promoter (mut) and treated with LzCD95L (Lz), anti-APO-1 (A), sCD95L, or TNFα (for 4 hrs) assayed over time. (**B**) Three day invasiveness assay of MCF7(FB) cells stimulated with 1 μg/ml LzCD95L, 1000 U/ml TNFα, or 1 μg/ml LzTRAIL. (**C**) MCF7 (FB) cells were incubated for the indicated times with LzCD95L or TNFα. Cell lysates were subjected to IKKβ immunoprecipitation followed by *in vitro* kinase assay (IKA) using GST-IκBα as the substrate. Phosphorylated IκBα was resolved and exposed to film. W, Western blot. (**D**) Jurkat cells deficient for RIP

expression stimulated with either anti-APO-1/zVAD-fmk (A), LzCD95L/zVAD-fmk (Lz) or TNFα for 4 hrs were subjected to EMSA. P, parental Jurkat cells, R, RIP deficient cells.

Fig. 5. All three MAP kinase pathways are activated by LzCD95L in MCF7(FB) cells and activation of Erk is involved in CD95 induced invasiveness. (**A**) MCF7(FB) cells were stimulated with LzCD95L for indicated times and lysates of the cells subjected to Western blot analysis for phosphorylation of indicated MAP kinases. Ratios of intensity of phosphorylated versus unphosphorylated kinase bands were determined by densitometry. (**B**) Kinetics of phosphorylation of p42/p44 Erk in LzCD95L treated MCF7(FB) cells. As indicated (30 min stimulated) cells were preincubated with the following inhibitors: 20 μM PD98059 (PD), 40 μM zVAD (Z) or 40 μM zIETD (I). (**C**) MCF7(FB) cells were pre-incubated for 3 hours with 140 μM of control-peptide (C) or NEMO binding domain(NBD)-peptide (N) and then stimulated for 2 hours with LzCD95L. Nuclear extracts were purified and subjected to an EMSA (top panel). In parallel cells were lyzed and phosphorylation of Erk was analyzed by Western blotting (bottom panels). (**D**) Invasiveness assay of MCF7(FB) cells preincubated with PD98059 or p38 inhibitor SB203580. Effectiveness of the SB inhibitor was tested using phosphospecific antibodies (not shown). (**E**) CAT reporter assay of LzCD95L stimulated MCF7(FB) cells following preincubation with the PD inhibitor.

Fig. 6. CD95 induced invasiveness requires caspase-8 activity. (**A**) Invasiveness assay of MCF7(FB) cells in the presence of zVAD-fmk (z) or zIETD-fmk (I). (**B**) κB driven CAT reporter assay of MCF7(FB) cells preincubated with zVAD-fmk (z) or zIETD (I). (**C**) Invasiveness assay of NCI60 cells stimulated through CD95 in the presence of 20 μM PD98059 (PD) or 40 μM zIETD-fmk (I).

Figure 1

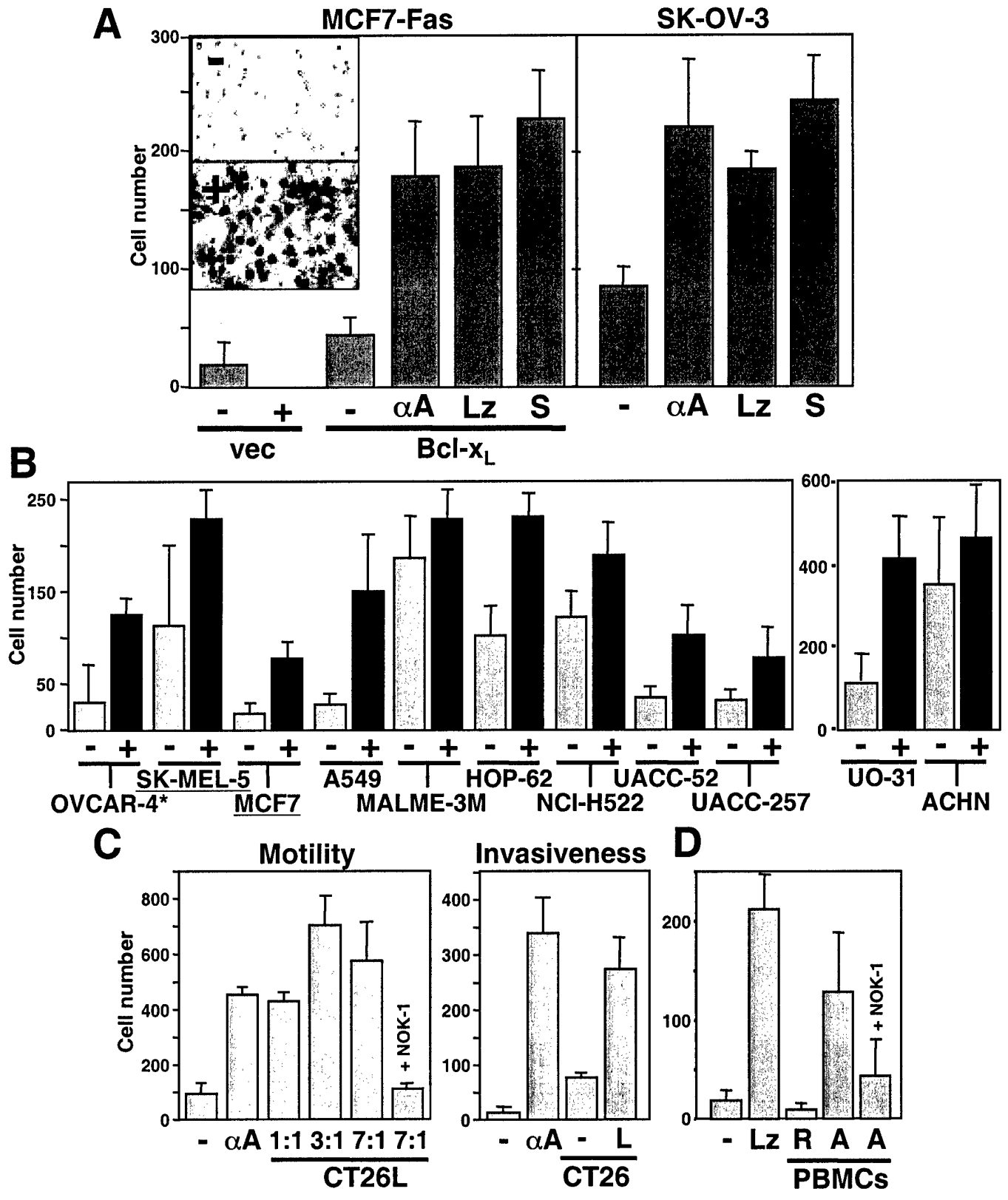
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Figure 2

A

Group I: Antiapoptotic	**A20** (5.3), **Bcl-x_L** (4.3), Akt3 (4), **cIAP-2** (3.7), Mcl-1 (3.5), **Bcl-xβ** (3.5), MEK2 (3.0), Survivin (2.3), **mMn-SOD** (2.3), HSP70 (2), **GADD45β** (2), **IEX-1L** (2)
Group II: Proproliferative	Brahm-related protein 1 (6.5), COX5b (5.3), COX5C (5.3), H2A (4.3), CKIγ (4.3), CARM1 (4), ATPase (lysosomal) (3.5), MEK5c (3.5), C/EBPδ (3.5)(2.3), H3A (2.8), Ferritin light chain (2.5), MAP3K12 (2.3) (2.7), CKII (2.1), Ribosomal proteins: S11 (9.1), S19 (6.5), P2 (6.1), S20 (5.3), L27a (4.6), L37a (4.6), L27 (4), S10 (3.5), L38 (2.8), L14 (2.6), mL63 (2.1), S20 (2), Protein translation: eIF5A (5.6), EF-1δ (4.3), eIF-2-α-kinase (2.1)
Group III: Tumor promoting	**ICAM1** (8), Wnt5a (7.5), **uPA** (4.3), MEF5 (3.5), Ephrin A1 (3.3), Sterolisomerase (3.2), GADD34 (2.5), methionine adenosyltransferase II, alpha (2.5), jagged 1 (2.3), **IL-8** (2)
Group IV: Other NF-kB targets	**IRF-1** (4.6), **IκBα** (4), **LTB** (3.3), **TNFα** (3), **p53** (2), **DR5** (2)
Group V: Other	3 cytoskeleton, 6 metabolism, 9 signaling, 5 chromatin binding, transcription factors, 23 ESTs and proteins of unknown function, 15 others

B

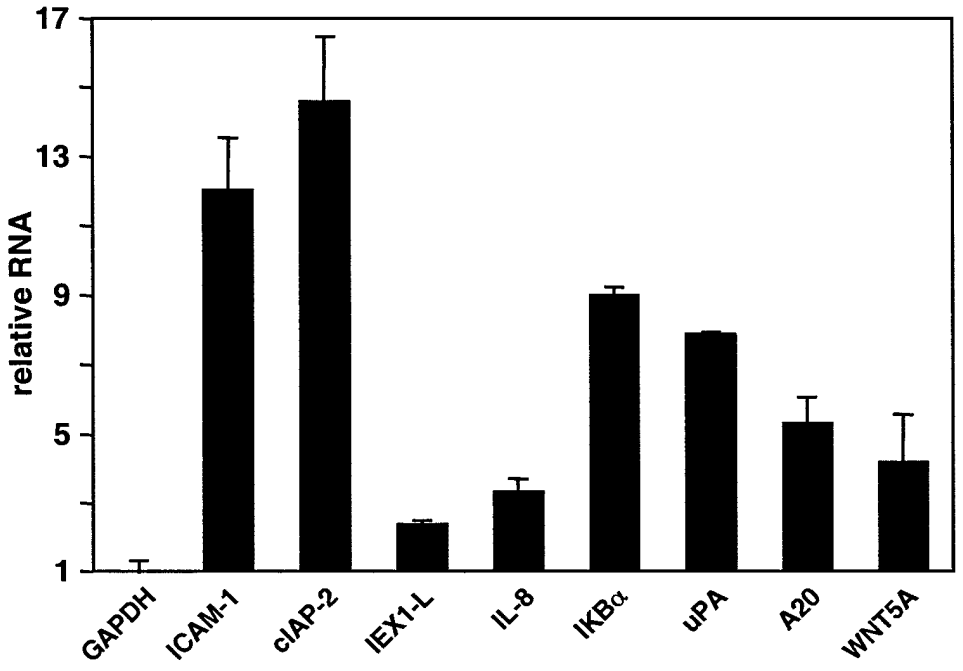

Figure 3

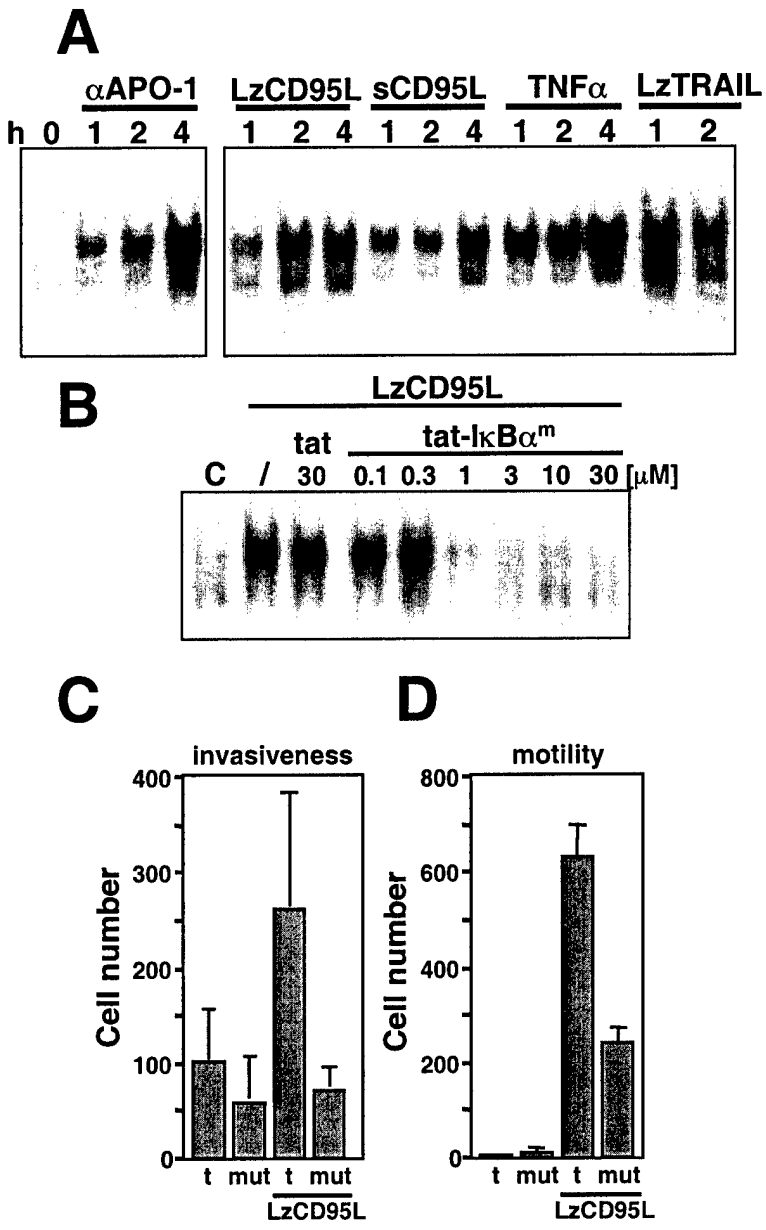
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Figure 4

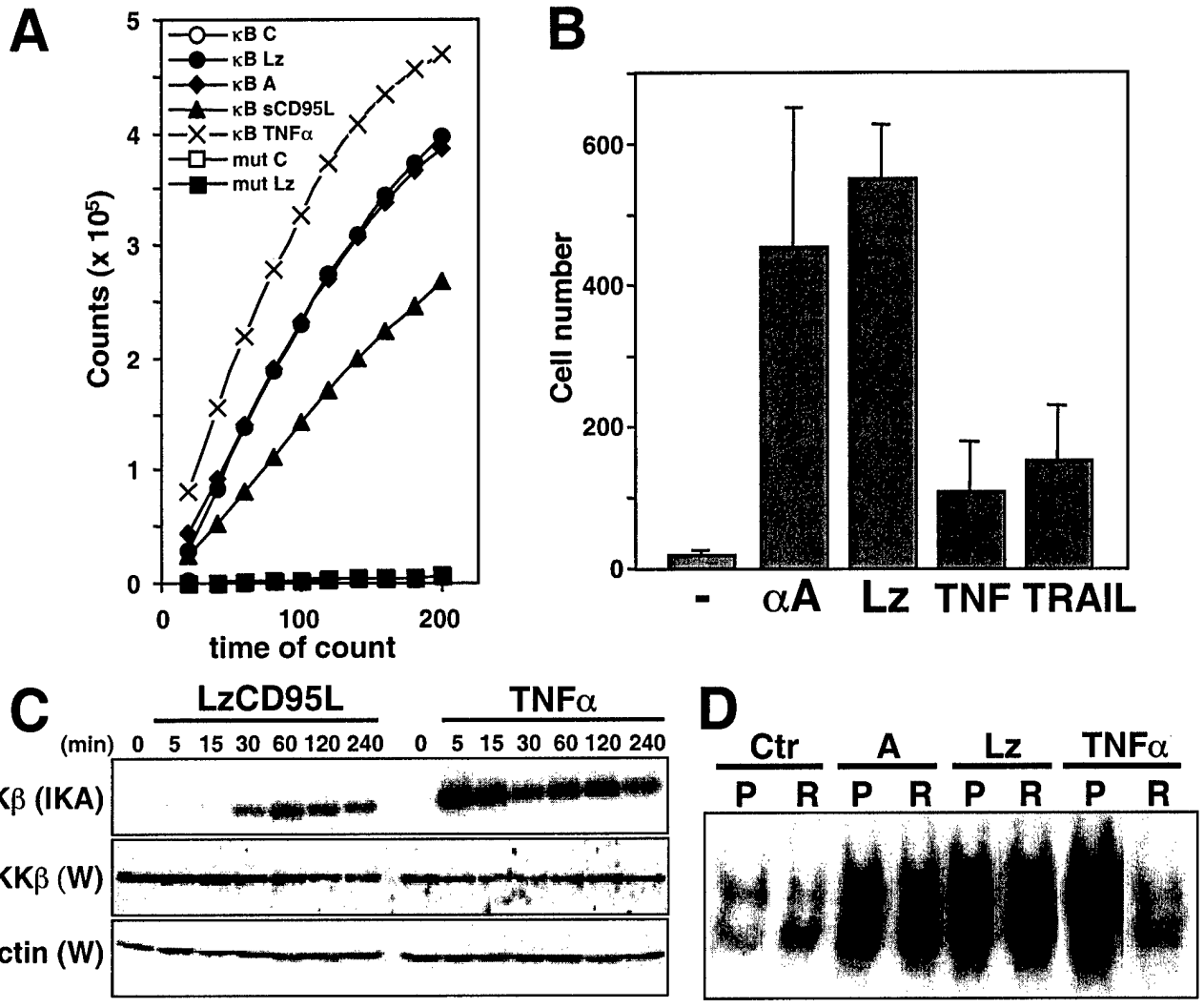
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Figure 5

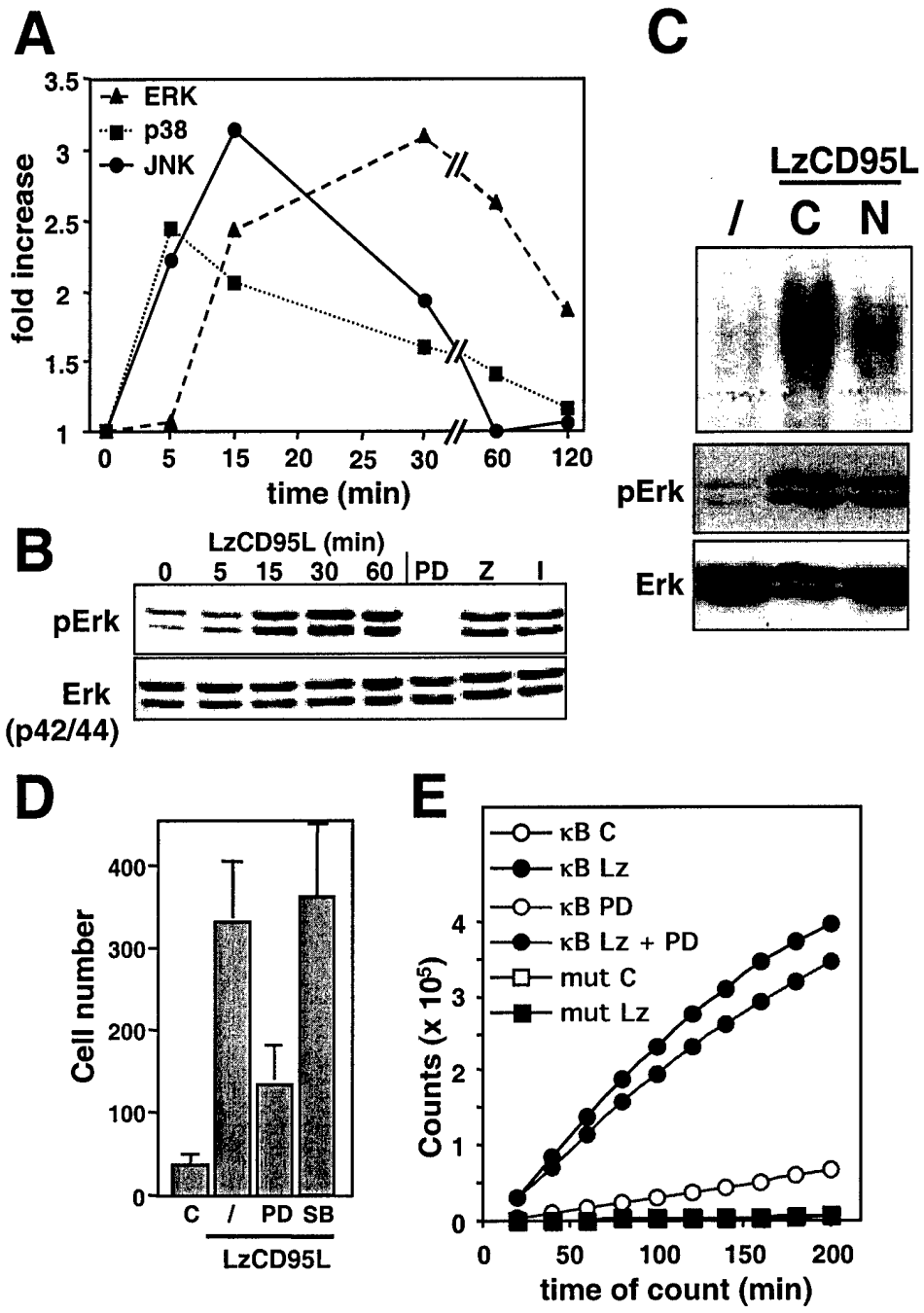
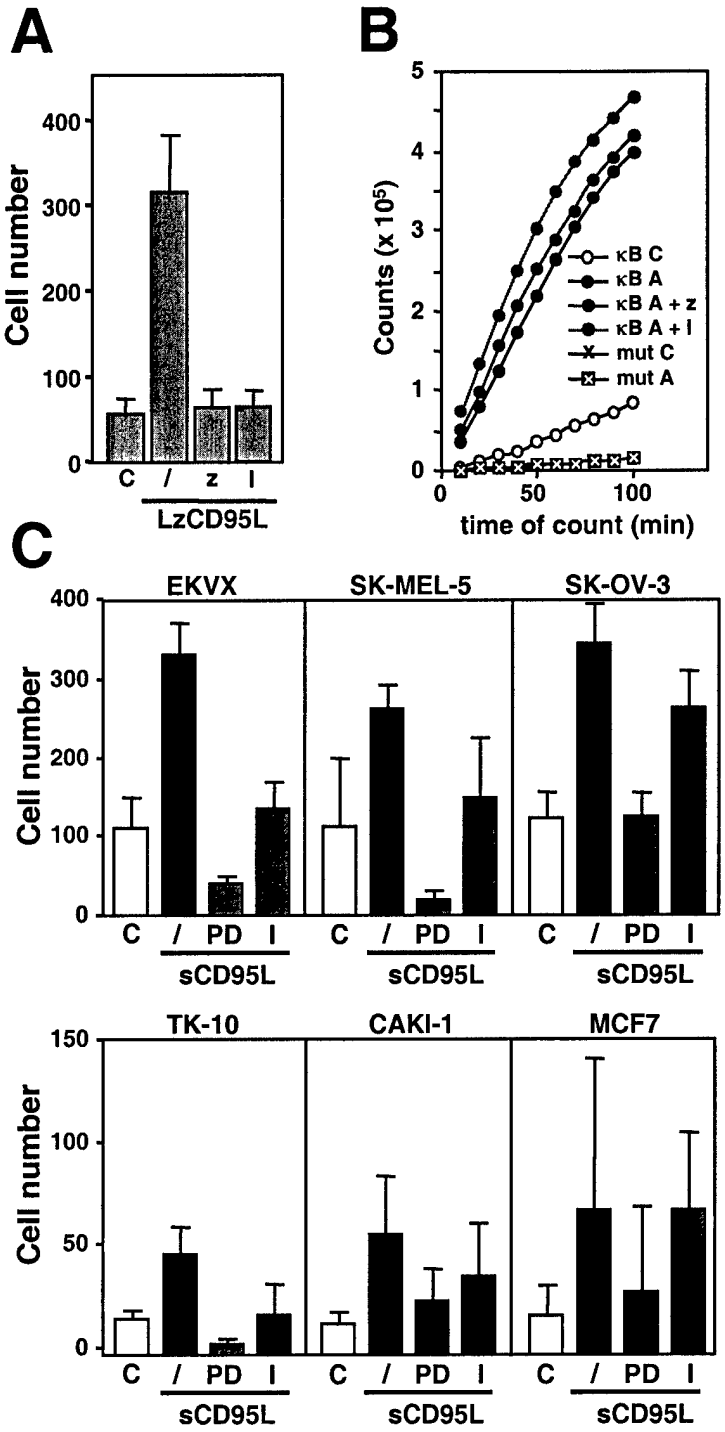

A
ERK
p38
JNK
fold increase
time (min)
B
LzCD95L (min)
0 5 15 30 60 PD Z I
pErk
Erk
(p42/44)
C
LzCD95L
/ C N
pErk
Erk
D
Cell number
C / PD SB
LzCD95L
E
κB C
κB Lz
κB PD
κB Lz + PD
mut C
mut Lz
Counts (x 10^5)
time of count (min)

Figure 6


A
Cell number
400
300
200
100
0
C / z I
LzCD95L
B
Counts (x 10^5)
5
4
3
2
1
0
0
50
100
time of count (min)
κB C
κB A
κB A + z
κB A + I
mut C
mut A
C
EKVX
SK-MEL-5
SK-OV-3
Cell number
400
300
200
100
0
C / PD I
sCD95L
TK-10
CAKI-1
MCF7
150
100
50
0

Legends to supplementary figures.

Fig. S1. Details of the gene chip analysis of MCF7(FB) cells treated with anti-APO-1 for 8 hrs. Genes in red are known NF-κB target genes. The position in the gene screen is given for each gene and its fold induction over unstimulated cells.

Fig. S2. Characteristics of activation of NF-κB by CD95 in MCF7(FB) cells. (**A**) EMSA analysis of MCF7(FB) cells left untreated or pre-treated with 1 μg/ml cycloheximide for one hour before a 4 hrs stimulation with anti-APO-1 (A) or control Ab (C). (**B**) Supershift EMSA was performed by incubating extracts from 4 hrs stimulated MCF7(FB) cells with indicated antibodies specific for NF-κB components. (**C**) MCF7(FB) cells were pre-incubated for 3 hours with 200 μM of control-peptide or NEMO binding domain(NBD)-peptide and then stimulated for 2 hours with the indicated stimulus. Nuclear extracts were purified and subjected to an EMSA. (**D**) Cells were treated as described in Figure S2C and for each sample, 5 μg of nuclear protein were loaded in a p65 ELISA (see Materials and Methods). NF-κB activity is expressed as the ratio of NBD-peptide treated cells relative to control-peptide treated cells x100.

Fig. S3. CD95 stimulation induces activation of NF-κB in the majority of CD95 resistant tumor cell lines. (**A**) CD95 apoptosis resistant cells from the NCI drug screening panel activated for four hours with the indicated stimulus. A, anti-APO-1; Lz, LzCD95L; S, sCD95L. (**B**) Prototype Type I cells H9 and SKW6.4 stimulated with sCD95L for 4 hrs unless otherwise indicated. (**C**) Type I cells from the NCI drug screening panel incubated without (-) or with (+) with sCD95L for 4 hrs. All cells were analyzed by EMSA.

Figure S1: Genes upregulated in MCF7-Fas-Bcl-x_L cells treated with anti-APO-1

Pos.	Fold ind.	Accession #	Gene cluster/Name
Antiapoptotic cluster			
15	5.28	AI738896	A20
23	4.29	NM_001191.1	Bcl-x_L
28	4.00	NM_006290.1	A20
33	4.00	AF135794.1	AKT3/protein kinase Bγ
35	3.73	U37546.1	inhibitor of apoptosis 2 (cIAP2)
40	3.48	H71805	myeloid cell leukemia sequence 1 (Mcl-1)
42	3.48	U72398	Bcl-x beta (bcl-x)
52	3.03	AI762811	mitogen-activated protein kinase kinase 2, MEK2
76	2.46	X15132.1	superoxide dismutase 2, mitochondrial
79	2.30	NM_001168.1	baculoviral IAP repeat-containing 5 (survivin)
84	2.30	W46388	superoxide dismutase 2, mitochondrial
107	2.00	NM_003897.1	immediate early response 3 (IEX-1L)
109	2.00	NM_005346.2	heat shock 70kD protein 1B
115	2.00	NM_015675.1	growth arrest and DNA-damage-inducible, beta (GADD45B)
Proproliferative cluster			
2	9.19	BF680255	ribosomal protein S11
9	6.50	BC000023.1	ribosomal protein S19
11	6.06	BC005354.1	ribosomal protein, large P2
13	5.66	NM_001970.1	eukaryotic translation initiation factor 5A
16	5.28	AI557312	cytochrome c oxidase subunit Vb
17	5.28	AF113008.1	ribosomal protein S20
20	4.59	BE737027	ribosomal protein L27a
21	4.59	BE857772	ribosomal protein L37a
24	4.29	AI335509	eukaryotic translation elongation factor 1 delta
29	4.00	BE312027	ribosomal protein L27
30	4.00	AA393940	eukaryotic translation initiation factor 5A
39	3.48	AW302047	ribosomal protein S10
54	3.03	BF541557	eukaryotic translation initiation factor 5A
59	2.83	BF690020	eukaryotic translation elongation factor 1 delta
64	2.83	AW303136	ribosomal protein L38
69	2.64	BC000606.1	ribosomal protein L14
75	2.46	AI345238	ferritin, light chain
78	2.30	BC000603.1	ribosomal protein L38
95	2.14	AF116615.1	heme-regulated initiation factor 2-alpha kinase
101	2.14	BF195165	mitochondrial ribosomal protein 63
120	2.00	AF113008.1	ribosomal protein S20
Tumor promoting/cancer related cluster			
5	8.00	NM_000201.1	intercellular adhesion molecule 1 (ICAM1/CD54)
6	7.46	NM_003392.1	wingless-type MMTV integration site family, member 5A (WNT5A)
8	6.50	AI608725	intercellular adhesion molecule 1 (ICAM1/CD54),
22	4.29	NM_002658.1	plasminogen activator, urokinase
37	3.48	U71088.1	mitogen-activated protein kinase kinase 5 (MEK5)
44	3.25	NM_004428.1	ephrin-A1
49	3.25	N58493	emopamil-binding protein (sterol isomerase)
70	2.46	NM_014330.2	growth arrest and DNA-damage-inducible 34, GADD34
74	2.46	AW301861	methionine adenosyltransferase II, alpha
81	2.30	U73936.1	jagged 1
110	2.00	NM_000584.1	interleukin 8
104	2.00	U83981	GADD34
121	2.00	U77914.1	jagged 1
Other known and putative NF-kB targets			
19	4.59	NM_002198.1	interferon regulatory factor 1 (IRF-1)
27	4.00	AI078167	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα)
45	3.25	NM_002341.1	lymphotoxin beta (TNF superfamily, member 3)
50	3.03	NM_000594.1	tumor necrosis factor (TNF superfamily, member 2), TNFα
66	2.64	NM_004556.1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (IkBε)
108	2.00	NM_000546.2	p53
116	2.00	AF016266.1	tumor necrosis factor receptor superfamily, member 10b TRAIL-R2/DR5

Figure S1: Genes upregulated in MCF7-Fas-Bcl-x_L cells treated with anti-APO-1

Other				Function
1	16.00	AI479277	EST	unknown
3	8.57	AA910371	calreticulin	protein folding
4	8.57	AK023817.1	EST	unknown
7	6.96	AF312230.1	histamine H4 receptor	
10	6.50	AI831675	SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	chromatin structure
12	6.06	AL050388.1	DKFZp564M2422	unknown
14	5.66	AK022217.1	Homo sapiens cDNA FLJ12155 fis	unknown
18	4.59	D29805.1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	required for synthesis of lactosylceramide which induces NF-kB
25	4.29	BC001629.1	histone family, member O	chromatine structure
26	4.29	AK025179.1	casein kinase 1, gamma 1	signaling
31	4.00	AA679297	death supressor WA1 (CIR2/CROC1/UEV1)	unknown
32	4.00	AL529396	coactivator-associated arginine methyltransferase-1 (CARM1)	methylates Arg 17 in histon 3, necessary for gene activation)
34	4.00	AA706035	EST	unknown
36	3.48	NM_005195.1	CCAATenhancer binding protein (CEBP), delta	transcription factor
38	3.48	BF061054	CCAATenhancer binding protein (CEBP), delta	transcription factor
41	3.48	AI252582	ATPase, H+ transporting, lysosomal (vacuolar proton pump) 9kD	metabolism
43	3.48	BF942161	EST	unknown
46	3.25	U20489.1	Human glomerular epithelial protein 1 (GLEPP1)	receptor tyrosine phosphatase
47	3.25	H51429	H2A histone family, member X	chromatin structure
48	3.25	AA156251	secreted protein of unknown function	unknown
51	3.03	AA724134	transducin (beta)-like 1	G protein coupled receptor
53	3.03	AA993683	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2 (8kD, B8)	oxidative phosphorylation
55	3.03	BE891920	actin related protein 2/3 complex, subunit 4 (20 kD) (Arp2/3 complex)	cytoskeleton
56	3.03	BG223334	Endonuclease G precursor	proapoptotic
57	3.03	AW392551	EST	unknown
58	3.03	AW152437	EST	unknown
60	2.83	BF339133	peroxisome biogenesis factor 10	protein involved in import of peroxisomal matrix proteins
61	2.83	AA292281	H3 histone, family 3A	chromatin structure
62	2.83	NM_030952.1	human SNARK kinase	signaling
63	2.83	NM_023922.1	taste receptor, type 2, member 14	
65	2.83	AF143877.1	Homo sapiens clone IMAGE:113431 mRNA sequence	unknown
67	2.64	NM_015887.1	putative peroxisome microbody protein 175.1	unknown
68	2.64	AI912086	Human DNA sequence from clone 1170K4 on chromosome 22q12.2-13.1.	unknown
71	2.46	NM_005419.1	signal transducer and activator of transcription 2, STAT2	transcription factor
72	2.46	NM_005538.1	inhibin, beta C	TGFβ homolog
73	2.46	U84138.1	RAD51 (S. cerevisiae)-like 1	DNA repair
77	2.30	L08599.1	cadherin 1, type 1, E-cadherin (epithelial)	cell adhesion
80	2.30	NM_006301.1	mitogen-activated protein kinase kinase kinase 12	activates JNK
82	2.30	U69563	Rho6/Rnd1	induces disassembly of actin stress fibers
83	2.30	AA551784	coactivator-associated arginine methyltransferase-1	unknown
85	2.30	AK024455.1	FLJ00047 protein	unknown
86	2.30	AF065241.1	Homo sapiens thioredoxin delta 3 (TXN delta 3)	redox signaling
87	2.30	S81916.1	phosphoglycerate kinase {alternatively spliced}	metabolism
88	2.30	AI127700	EST	unknown
89	2.14	AI949392	Semaphorin 4C	regulates axon guidance
90	2.14	NM_015596.1	kallikrein 13 (prorenin processing enzyme)	serine protease involved in carcinogenesis
91	2.14	NM_031308.1	epiplakin 1 (EPPK1)	cytoskeleton
92	2.14	AK024505.1	f-box and leucine-rich repeat protein 11	F-box protein
93	2.14	U05598.1	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	metabolism
94	2.14	M37435.1	colony stimulating factor 1, CSF-1	cytokine
96	2.14	AF336878.1	FKSG51 mRNA	unknown
97	2.14	AA909765	KIAA0906 protein	unknown
98	2.14	AI348935	calreticulin	protein folding
99	2.14	S68290.1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	metabolism
100	2.14	AJ224869	CXCR4 (fusin)	chemokine receptor
102	2.14	AW515443	similar to rat nuclear ubiquitous casein kinase 2	signaling
103	2.14	AB004064.1	tomoregulin	transmembrane protein of unknown function
105	2.00	AI201594	EST	unknown

Figure S1: Genes upregulated in MCF7-Fas-Bcl-x_L cells treated with anti-APO-1

106	2.00	NM_003407.1	zinc finger protein homologous to Zfp-36 in mouse	posttranscriptionally regulates TNFα
111	2.00	NM_001924.2	growth arrest and DNA-damage-inducible, alpha (GADD45A)	DNA damage response
112	2.00	NM_001353.2	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	metabolism
113	2.00	NM_004209.2	synaptogyrin 3	unknown
114	2.00	AJ223333.1	DNA (cytosine-5-)-methyltransferase 2	DNA methylation
117	2.00	M33376.1	pseudo-chlordecone reductase	unknown
118	2.00	AW131783	nucleolar protein family A, member 2 (HACA small nucleolar RNPs)	unknown
119	2.00	AL390149.1	DKFZp547B026	unknown

Figure S2

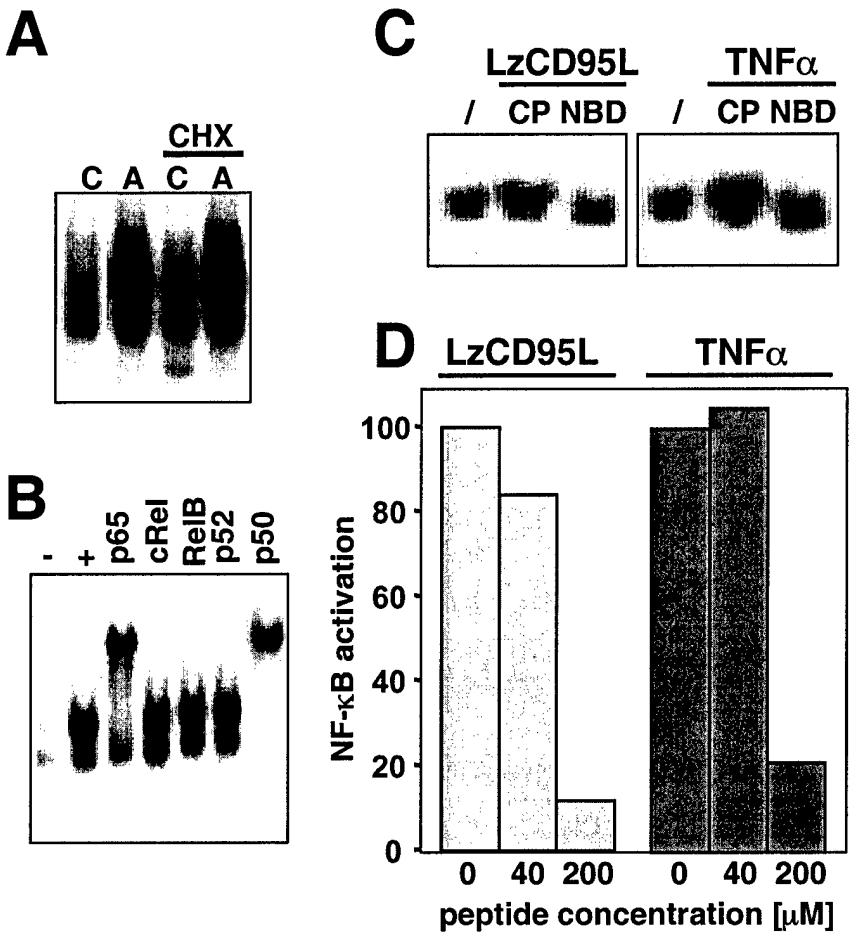
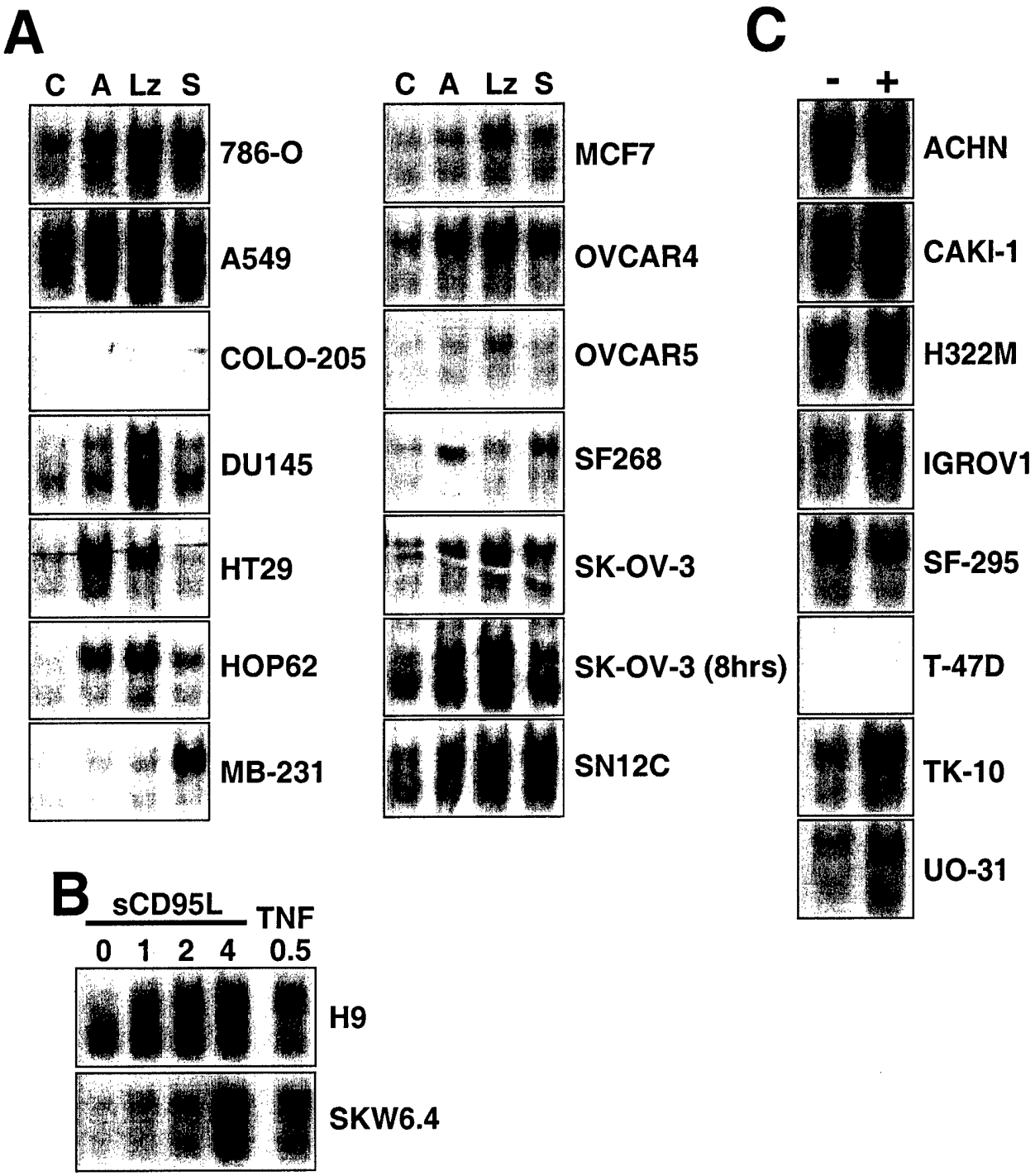

A
CHX
C A C A
B
- + p65 cRel RelB p52 p50
C
LzCD95L
TNFα
/ CP NBD / CP NBD
D
LzCD95L
TNFα
NF-κB activation
100
80
60
40
20
0
0 40 200 0 40 200
peptide concentration [μM]

Figure S3


A
C A Lz S
786-O
A549
COLO-205
DU145
HT29
HOP62
MB-231
C A Lz S
MCF7
OVCAR4
OVCAR5
SF268
SK-OV-3
SK-OV-3 (8hrs)
SN12C
C
- +
ACHN
CAKI-1
H322M
IGROV1
SF-295
T-47D
TK-10
UO-31
B
sCD95L
TNF
0 1 2 4 0.5
H9
SKW6.4

Supplementary data section; methods:

Gene chip analysis and real time PCR

10^7 MCF7(FB) cells were stimulated with 1μg/ml anti-APO-1 for 8 hours and mRNA isolated by the TRIZOL method with one additional phenol/choloroform/isoamyl alcohol purification prior to ethanol precipitation in 0.3 M sodium acetate. Integrity of RNA was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and purity/concentration was determined using a GeneSpec III (Miraibio). The target preparation protocol followed the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA). Briefly, 10 μg of total RNA was used to synthesize double-stranded cDNA using the Superscript Choice System (Life Technologies). First strand cDNA synthesis was primed with a T7-(dT_{24}) oligonucleotide. From the phase-log gel-purified cDNA, biotin-labeled antisense cRNA was synthesized using BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). After precipitation with 4 M lithium chloride, 12 μg of fragmented cRNA was hybridized to Human 133A Arrays for 16 hours at 45° C and 60 rpm in an Affymetrix Hybridization Oven 640. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 400 using the Affymetrix GeneChip protocol and then scanned using the Affymetrix Agilent GeneArray Scanner. The acquisition and initial quantification of array images was performed using the Affymetrix Microarray Suite Version 5.0 (MAS 5.0) with

the default analytic parameters. Subsequent data analysis was done as follows: 1) Evaluation of hybridization quality. This was achieved first by examining Report file for housekeeping gene hybridization (detection calls for housekeeping genes should be present for each of the hybridizations), Spike control hybridization (detection calls should be present most of time for BIOB and all the time for BIOC), percentage of genes called present (30% - 50%), 5' to 3' ratio (to validate IVT procedure), signal to background ratio and scale factor ratio and then by dChip analysis for regional image contamination and/or sample contamination. Arrays with array outliers ≥ 15% were discarded. 2) Data filtration. We used a two-step filtration strategy. The first step was to filter genes with signal intensity in all samples ≤ 100 intensity units. The rationale for choosing 100 as the first-step cut-off intensity was based on our observation that the final concentration of spike control BioB in the hybridization mix is 1.5 pM, which is equivalent to 1-3 RNA molecules per cell, but the signal intensity of BioB is normally above 100 when the global scaling target signal is set as 500 (Affymetrix Microarray Suit default setting). Furthermore, over 99% of the genes with signal intensity ≤ 100 are called absent by MAS 5.0. The second step filtration was to remove the genes that receive an "absent" call for all chips. 3) Identification of significantly differentially expressed genes with variable thresholds. Given the fact that the fold-change variability is a function of the signal intensity, we analyzed 65 replicate Affymetrix chip-chip comparisons and determined a series of signal-dependent thresholds for declaring

significant changes (a detailed description of this method can be found at: http://fgf.bsd.uchicago.edu). These thresholds offer user-friendly features like traditional fold-change thresholds but provide a 95% confidence interval like other statistical treatments. The genes listed in Figure S1 were selected on the basis of these variable fold-change thresholds. For real-time PCR total RNA was isolated with 400 μl Trizol-LS. Reverse-transcribed cDNA was made from 1 μg of each RNA sample and was used for all subsequent real-time PCR reactions. Specific primer sequences are available upon request. Cycles were run on an Applied Biosystems ABI 7700, and CYBR green was used for fluorescent detection of dsDNA, as per the manufacturer's instructions. YBR Green PCR Core Reagents purchased from Applied Biosystems (Foster City, CA) Normalization of the initial RNA composition was calculated from concurrent runs with primers specific for actin. Triplicates were performed with each primer set for each RNA sample. The mid-linear range was used to establish the threshold for each oligonucleotide set. After normalization and the calculation of differences within each time-point experiment subset, all data from each time point were pooled to calculate differences.

Generation of a soluble membrane permeable NF-κB inhibitor

GST-TAT and GST-IκBαM-TAT were produced by using the pGEX-3X-TAT and pGEX-3X-IκBαM-TAT plasmids, respectively. To obtain pGEX-3X-IκBαM-TAT, the full-length

IκBαM coding sequence (without stop) was isolated by polymerase chain reaction (PCR) from pcDNA-IκBαM (De Smaele *et al.,* 2001) using the following primers: 5'-GGAAGGATCCCAA**TG**TTTCAGCCAGCTGGGCACGG-3' and 5'-GGTAAGCTTCCTAATGTCAGACGCTGGCCTCCAAAC-3' (BamHI and HindIII sites incorporated into sense and anti-sense primers, respectively, are underlined; the initiating ATG is in bold). PCR products were then digested with BamHI and HindIII and ligated along with a HindIII-XhoI linker into pGEX-4T opened with BamHI and XhoI. The linker encoded a HIV-TAT cell-permeable peptide and was generated by annealing the following oligonucleotides: 5'-AGCTTACCCGGGGGCCGGAAGAAGCGGCGGCAGCGGCGGCGG**TGA**C-3' and 5'-TCGAGTCACCGCCGCCGCTGCCGCCGCTTCTTCCGGCCCCCGGGTA-3' (partial HindIII and XhoI sites incorporated into sense and anti-sense primers are underlined; the stop codon is in bold). The resulting pGEX-4T-IκBαM-TAT plasmid encoded GST followed sequentially by a precisions protease cleavage site, IκBαM, and TAT. To obtain pGEX-3X-IκBαM-TAT, the IκBαM-TAT coding sequence was excised from pGEX-4T-IκBαM-TAT by using BamHI and NotI (with the latter site being located in pGEX-4T immediately downstream of XhoI) and introduced into the BamHI and EcoRI sites of pGEX-3X along with the following NotI-EcoRI oligonucleotide linker: 5'-GGCCGCGATATCAAGCTTACG-3' (sense) and 5'-

AATTCGTAAGCTTGATAGATCTATCGC-3' (anti-sense; partial NotI and EcoRI sites incorporated into sense and anti-sense primers are underlined). pGEX-3X-TAT was obtained by excising IκBαM-TAT from pGEX-3X-IκBαM-TAT using BamHI and SmaI and ligating the insert-less vector after filling the BamHI site by Klenow reaction. Clones were confirmed by sequencing and appropriate restriction digestions. Recombinant GST-TAT and GST-IκBαM-TAT proteins were purified from BL21 *E. coli* lysates by using standard glutathione affinity chromatography and dialyzed against 5 mM phosphate buffer (pH 7.6). Protein purity (>95%) and integrity were assessed by SDS gel electrophoresis followed by Coomassie brilliant blue staining.

NF-κB ELISA

Cells were pre-incubated with NBD-peptide or control peptide (BIOMOL Research Laboratories, Inc.) for 3 hours at 37°C and then stimulated with the indicated stimuli for 2 hours. Nuclear extracts were purified, as described above, and the quantity of p65 was measured in a TransAM NF-κB ELISA following the instructions of the manufacturer (Active Motif). The OD was read at 450 nm using kinetic Microplate reader (molecular devices, CA). For each well, the Background was subtracted using the lysis buffer only. NF-κB activity is expressed as the ratio of NBD-peptide treated cells relative to control peptide treated cells.

Electrophoretic mobility-shift assay (EMSA)

1.5-2x10^6 cells were washed in ice cold PBS and resuspended in ice cold modified lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 0.5 mM PMSF, containing protease inhibitor cocktail (Sigma), incubated on ice for 5 min, and centrifuged at 40,000rpm for 30min at 4°C. Alternatively, nuclear extracts were isolated as described previously (Jobin *et al.,* 1997). Protein concentration was determined by the Bradford method and the resulting extracts were stored at –80C. EMSAs were performed by incubating 5 μg of cell extract (or 4μg of nuclear extract) in 20 mM HEPES pH 7.9, 20% glycerol, 100mM KCl, 0.2mM EDTA, 0.5 mM DTT, 0.5 mM PMSF containing 2mg/ml BSA, and 1mg/ml poly dIdC. The extracts were incubated for 15 min at room temperature followed by addition of ^{32}P-labeled NF-κB probe (PD-1, Franzoso *et al.,* 1992) for 10 min. Samples were analyzed by 4% native PAGE in 0.25x TBE buffer, the gels dried and subjected to autoradiography. Supershift analysis during an EMSA was performed by incubating cell extracts with antibodies to NF-κB subunits for 20 min on ice prior to the addition of the probe. The anti-p50 antibody was from Santa Cruz, the antibody against p65 has been described before (Franzoso *et al.,* 1992) and the anti-p52, anti-RelB and anti-c-Rel antibodies were a generous gift from Dr. U. Siebenlist (NIH).

CAT Assay

Cells were transfected with HIV-κB-CAT CAT reporter driven by the κB sites in the HIV LTR (Franzoso *et al.,* 1992) or a mutated promoter using Polyfect from Qiagen (Maryland) according to manufacturers protocols. 1 x 10^5 cells were harvested after 16-24 hours and stimulated with 1 μg/ml anti-APO-1, LzCD95L, sCD95L or recombinant human TNFα (Peprotech). Following eight hour stimulation cells were washed in PBS and resuspended in 25μl of 0.1M Tris pH 7.8. Cells were freeze/thawed for three cycles and spun for 15 min at 14,000 rpm at 4°C. Extracts were incubated at 70°C for 10 min and incubated at room temperature for 10 min. 100 μl of 3 mg/ml chloramphenicol in 0.1 M Tris pH 7.8 containing 0.5 μCi of [^{3}H]Acetyl CoA was added to the extract followed immediately by 3 ml of Econofluor scintillation fluid. Radioactivity was read in a TriCarb Liquid Scintillation Analyzer every 10 minutes for 10 readings.

References to supplementary methods:

De Smaele,E., Zazzeroni,F., Papa,S., Nguyen,D.U.,, Jin,R., Jones,J., Cong,R. and Franzoso,G. (2001) Induction of gadd45β by NF-κB downregulates pro-apoptotic JNK signalling. *Nature,* **414**, 308-313.

Franzoso,G., Bours,V., Park,S., Tomita-Yamaguchi,M., Kelly,K. and Siebenlist,U. (1992) The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. *Nature*, **359**, 339-342.

Jobin,C., Haskill,S., Mayer,L., Panja,A.,and Sartor,R.B. (1997) Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. *J. Immunol.* ,**158**, 226-234.